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Award Number: DAMD17-02-1-0023

TITLE: Regulation of TRAIL-Medicated Apoptosis in Prostate Cancer by Overexpression of XIAP

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REPORT DATE: January 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)* 01-01-2006	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 15 DEC 2001 - 14 DEC 2005

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Regulation of TRAIL-Medicated Apoptosis in Prostate Cancer by Overexpression of XIAP	
	5b. GRANT NUMBER DAMD17-02-1-0023
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Benjamin Bonavida, Ph.D.	
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
The University of California Los Angeles, CA 90024-1406	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Patients with prostate cancer (CaP) develop resistance to conventional therapies and alternative therapies, such as immunotherapy, are being actively considered. TRAIL is selectively cytotoxic to tumor cells and minimally cytotoxic to normal tissues and is a candidate for immunotherapy. CaP cells, however, are resistant to TRAIL due to antiapoptotic mechanisms such as overexpression of XIAP. This proposal investigated the mechanism by which XIAP regulates resistance to TRAIL and the findings demonstrate that TRAIL resistance is regulated by the expression of the TRAIL receptor DR5. The regulation of DR5 was found to be under the control of the transcription repressor YY1. Inhibition of NF-κB inhibited both YY1 and XIAP and sensitized the cells with TRAIL-induced apoptosis. We demonstrate that YY1 regulates the transcription of DR5 via a YY1 DNA-binding site on the DR5 promoter. The clinical importance of XIAP expression in prostate cancer tissue was examined in tissue microarrays and we demonstrate its prognostic significance in prostate cancer patients. This also correlated with the overexpression of YY1 which also showed its prognostic significance in prostate cancer patients. In addition, we have demonstrated that cytokines derived from prostate cancer and/or from the tumor microenvironment regulate the constitutive activation of NF-κB and downstream XIAP and YY1 and regulate resistance to TRAIL. Overall, our findings identify new therapeutic targets for TRAIL.

15. SUBJECT TERMS
TRAIL, signaling, XIAP, apoptosis, NF-κB, sensitization, Bcl-xL, Immunotherapy, cytokines, drugs

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	363	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

Prostate cancer (CaP) is the most common malignancy and age-related cause of cancer deaths in American males. Treatments for metastatic prostate cancer include hormonal ablation, chemotherapy, and combination therapies. These treatments are aimed at inhibiting tumor growth and at also inducing apoptosis. Unfortunately, hormonal therapy is always followed by the relapse of an aggressive androgen-independent disease that is insensitive to further hormonal manipulation or to treatment with conventional chemotherapeutic drugs. The underlying mechanisms by which the prostate tumor cells develop resistance to hormonal/drug-mediated effects are poorly understood. It is probable that the tumor cells acquire mechanisms that resist androgen ablation/drug-mediated apoptosis. Failure to eradicate advanced resistant tumors with conventional therapies has led to the exploration of novel therapeutic approaches such as immunotherapy.

Immunotherapy is generally aimed at the generation of anti-tumor cytotoxic lymphocytes that can recognize and eradicate the drug-resistant tumor cells. Also, immunotherapy is predicated on the notion that all drug-resistant tumors should succumb to cytotoxic lymphocyte-mediated killing. Tumors that develop anti-apoptotic mechanisms to escape drugs/radiation-mediated apoptosis may also develop cross-resistance to apoptosis mediated by cytotoxic lymphocytes. We and others have reported that many types of drug-resistant tumor cells including CaP cells are also resistant to immune-related cytotoxic mechanism of killing. Thus, the development of immune resistance by drug-refractory tumors may hinder the therapeutic effect of immunotherapy. However, if the tumor can be sensitized to overcome resistance, the combination of sensitization and immunotherapy may be more efficacious. The underlying molecular mechanisms of sensitization to immunotherapy are therefore important to explore for the identification of gene targets involved in resistance and hence develop new means to modify these targets and reversing immune resistance.

The inhibitor of apoptosis proteins (IAPs) represent a family of endogenous caspase inhibitors that share a conserved structure known as the BIR domains (Reed, 2001). Eight encoding IAPs genes are found in the human genome, and some of these are overexpressed in cancers (Ferreira *et al.,* 2001; Hofmann *et al.,* 2002; Tamm *et al.,* 2000). These proteins are involved in maintaining tumor cell survival or for regulating resistance to apoptosis induction by various therapeutics (Bilim *et al.,* 2003). The X-chromosome linked IAP (XIAP) is the best characterized of the IAP family members in terms of its caspase inhibitory mechanism. The XIAP protein contains three BIR domains. XIAP levels have been shown to be pathologically elevated in many acute and chronic leukemia, prostate cancer, lung cancer, and other tumor (Byrd *et al.,* 2002; Ferreira *et al.,* 2001; Hofmann *et al.,* 2002; Schimmer *et al.,* 2003; Seligson *et al.,* 2006). Schimmer *et al.,* (2004) have recently reported that small molecule antagonists of apoptosis suppress XIAP function and exhibit a broad anti-tumor activity and such molecules are considered as targets for cancer therapy.

The TNF ligand superfamily serves as an important role in the host immune defenses against cancer as an inducer of apoptosis in tumor cells. Apo2L/TRAIL recently has drawn interest as a potential effective anti-tumor therapeutic agent in a variety of cell lines since it is selectively cytotoxic against transformed cells and not against the majority of normal cells. Our recent findings have demonstrated that drug-resistant CaP cells are resistant to TRAIL and also demonstrated that chemotherapeutic drugs (e.g. Act D, ADR and VP-16) sensitize CaP cells to TRAIL-mediated apoptosis (Zisman et al, 2001; Ng et al., 2002). Unlike a recent study showing

FLIP overexpression is responsible for TRAIL (Fulda et al., 2000), we have also identified a member of inhibitor of apoptosis proteins (IAPs), XIAP, as responsible for resistance in CaP. Therefore, this project is designed to investigate the role and regulation of XIAP overexpression in CaP in order to find new means to inhibit its expression by new agents and reverse TRAIL resistance for clinical use.

Our proposed studies are relevant to the success of CaP immunotherapy. Accumulating evidence suggest overexpression of IAPs, particularly XIAP, is a cause of apoptosis dysfunction in cancer cells (Holcik et al., 2000; Kitada et al., 2000). For instance, Tamm et al. (2000) reported the relevance of XIAP *in vivo* responses to cytarabine in AML. Patients with lower XIAP protein had significantly longer survival and a tendency towards longer remission duration than those with higher levels of XIAP. In prostate cancer, we identified XIAP as and important anti-apoptotic gene product that regulates TRAIL apoptosis.

In this grant application, we have proposed to investigate the following aims: 1) The role of XIAP in protecting CaP cells from TRAIL-mediated-apoptosis. Examination of the direct role of XIAP by transfection utilizing the newly reported IAP inhibitor Smac/DIABLO and by XIAP-antisense. The sensitivity to TRAIL and signaling pathway for apoptosis in transfectants and normal CaP will be compared. Further, we proposed to correlate the expression of both XIAP and Smac/ DIABLO in freshly derived normal, benign, and human prostate tumor cells at different stages of the disease and establish correlations with prognosis. 2) The role of constitutively activated NFκB (survival factor) in the regulation of both resistance to TRAIL and XIAP-expression. We proposed to examine the role of NFκB in the regulation of XIAP expression and TRAIL sensitivity. 3) The roles of constitutive and exogenous TNF-α and IL-6 in the regulation of NFκB, XIAP expression, and sensitivity to TRAIL.

Body

 The following tasks were proposed for investigation:

Task 1: The role of XIAP in protecting CaP cells from TRAIL-mediated apoptosis
Task 2: Regulation of XIAP by NFκB and NFκB regulation of XIAP
Task 3: To determine the role of endogenous TNF-α and IL-6 in the regulation of XIAP and resistance to TRAIL
      We have investigated all of the above proposed tasks and several reports emanated from our findings. Further, we have made several novel findings and have identified new gene products that regulate TRAIL sensitivity in prostate cancer cells. The following summarizes our progress to date.

      We have proposed to examine in Task 1 the direct role of XIAP in the regulation of TRAIL resistance by transfection experiments utilizing the newly reported IAP inhibitor, Smac/DIABLO and by XIAP antisense. We have also proposed to examine the sensitivity and signaling to TRAIL in the transfectants. We have completed the studies with the transfection of CaP cells with Smac/DIABLO and the studies have been reported in two publications (Ng, et al. 2002; Ng and Bonavida, 2002a). Briefly, the studies provided novel findings in the mechanism of XIAP regulation of TRAIL resistance. Noteworthy, we show in CaP cells that TRAIL signaling results in the loss of mitochondrial potential, the release of cytochrome c and Smac/DIABLO into the cytoplasm in the absence of caspase activation due to overexpression of XIAP. However, inhibition of XIAP by transfection with Smac/DIABLO (or treatment with Actinomycin D, which inhibits XIAP expression) allowed the TRAIL signaling to proceed to activation of caspase 9 and 3 and induction of apoptosis. These findings demonstrated that two complementary signals are involved to overcome prostate cancer as well as possibly other cancers to TRAIL-induced apoptosis. The two signal model has been proposed in the studies that we have reported and has also been detailed in a recent review that was published in *Advances in Cancer Research* (Ng and Bonavida, 2002b).

      In Task 1 we have also proposed to examine the expression of XIAP and Smac/DIABLO in freshly derived normal, benign, and prostate tumor cells at different stages and grades and correlation with prognosis. We have examined the expression of Smac/DIABLO in tumor cell lines by immunohistochemistry (IHC). We have established the optimal conditions and specificity of the antibody against Smac/DIABLO and demonstrated successfully that the antibody is applicable for IHC in addition to its usage for Western blot in our publications.
Due to limitations in CaP tissue microarrays, we first examined the clinical role of XIAP overexpresssion in such microarrrays.

 Prognostic significance of XIAP expression in CaP tissue microarrays
      Our findings have established that overexpression of XIAP in CaP is associated with both chemoresistance and immune resistance to TRAIL-induced apoptosis. We hypothesize that overexpression of XIAP in human CaP tissues may be associated with resistance and thus, may be of prognostic clinical significance. We examined the expression of XIAP by immunohistochemistry using CaP tissue microarrays and the data were statistically analyzed. The findings demonstrate that overexpression of XIAP is of prognostic significance in a subset of patients with prostate cancer. A manuscript has been completed and will be submitted shortly (Seligson et al., 2006; appendix). The abstract is highlighted below.

Abstract

Objective: The X-linked Inhibitor of Apoptosis (XIAP), a member of the family proteins, has been linked to tumor cell survival and drug resistance by direct blockade of caspase-mediated ~~extrinsic~~ apoptotic pathways. Thus, XIAP status may help predict prostate cancer recurrence and clinical response to therapies relying on unencumbered apoptotic machinery. It is therefore important to validate the foundational protein expression patterns of XIAP and examine its prognostic implications in human prostate cancer.

Methods: Immunohistochemistry was performed on tissue microarrays constructed from paraffin embedded primary prostate cancer specimens from 226 hormone naïve patients who underwent radical retropubic prostatectomy. 223 cases provided informative epithelium for XIAP analysis encompassing 1,107 total tissue microarray spots including morphologically normal prostate (NL; n=252), benign prostatic hyperplasia (BPH; n=122), prostatic intraepithelial neoplasia (PIN; n=48) and invasive prostate cancer (Cancer; n=685). XIAP expression was scored in a semi-quantitative fashion using an integrated intensity measure (0.0-3.0). The protein expression distribution was examined across the spectrum of epithelial tissues and its association with standard clinicopathological covariates and tumor recurrence was examined in 192 outcome-informative patients.

Results: The mean XIAP expression was significantly higher in prostate cancer (intensity = 1.32) compared to PIN (intensity = 1.08; p=0.019), normal (intensity = 0.78; p<0.0001), and BPH (intensity = 0.57; p<0.0001). 69% of BPH stained negatively to weakly (intensity <1.0), 53% of normal, 37% of PIN and only 26% of prostate carcinomas. With XIAP expression dichotomized at an intensity of 1.8, XIAP is an independent predictor of tumor recurrence in multivariate Cox proportional hazards analysis in all patients (P = 0.0025; HR = 8.92; 95% CI = 2.16-38.86), as well as after substratifying by Gleason score (P = 0.010; HR = 6.61; 95% CI = 1.57-27.89 for high Gleason score [7-10] cases). In patient substrata with low Gleason score tumors [2-6], no patients (0%) with an XIAP intensity > 1.8 (n=23) experienced tumor recurrence, while 26% with low XIAP (n=89) recurred. Patients with high grade or non-organ confined tumors with high XIAP have a lower risk of recurrence as a group than any patients whose tumors express low XIAP, even those of low grade or that are organ confined. These data are consistent with findings in a recent report (Krajewska 2003).

Conclusions: XIAP is expressed at higher levels in prostate cancers compared to matched normal tissues. High XIAP expression is strongly associated with a reduced risk of tumor recurrence, and is not directly associated with Gleason score, tumor stage, capsular involvement or preoperative PSA status, suggesting that it is a novel prognosticator and a potential target for prostate cancer diagnosis and therapy. Based on these results, patients with lower XIAP expression in tumors are most in need of therapeutic intervention and may also be most responsiveness to chemotherapeutic and death receptor targeted therapies.

In task 2 and 3 we have proposed to investigate the role of NF-κB and TNF-α in the regulation of XIAP.

1. Role of constitutive NF-κB activity and downstream anti-apoptotic gene expression (XIAP and Bcl-$_{xL}$) in the regulation of TRAIL resistance.

We and others have also demonstrated that prostate cancer cell lines exhibit constitutively active nuclear factor kappa B (NF-κB) (Suh *et al.,* 2002; Huerta-Yepez *et al.,* 2004). NF-κB regulates

the transcription of many anti-apoptotic gene products, including XIAP and Bcl-$_{xL}$. We examined the role and mechanism of NF-κB-induced resistance to TRAIL apoptosis. We used the nitric oxide donor DETANONOate and the NF-κB inhibitior Bay 11-7085 to inhibit NF-κB activity, and treated PC-3 cells resulted in downstream inhibition of both XIAP and Bcl-$_{xL}$ expression. The inhibition of NF-κB resulted in sensitization to TRAIL apoptosis. Further, the role of Bcl-$_{xL}$ in the regulation of TRAIL resistance was corroborated by the use of the chemical inhibitor 2-methoxyantimycin A which sensitized PC-3 cells to TRAIL-induced apoptosis. We further examined the apoptotic-signaling pathways following treatment of PC-3 cells with the combination of NF-κB inhibitors and TRAIL, and demonstrate that the combination, but not single agents alone, activate the mitochondrial pathway and the activation of caspases 9 and 3 and the induction of apoptosis. The above findings have been recently reported (Huerta-Yepez *et al.*, 2004).

2. Regulation of prostate carcinoma cell line resistance to TRAIL via upregulation of DR5 expression.

We have previously reported that treatment of CaP with nitric oxide donors or inhibitors of NF-κB resulted in sensitization to TRAIL-induced apoptosis (Huerta-Yepez *et al.,* 2004). We examined the mechanism of resistance by delineating putative transcription factors, aside from NF-κB, that may be involved in DR5 transcription. We demonstrate, by using various constructs of the DR5 promoter, that deletion in the promoter of a region with a putative YY1 DNA binding site resulted in significant augmentation of luciferase activity and suggested that YY1 may negatively regulate DR5 transcription. This finding was corroborated by demonstrating that mutation in the DNA binding site also resulted in augmentation of luciferase activity. In addition, inhibition of YY1 via chemical modification with nitric oxide or by transfection with sIRNA for YY1 resulted in both upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. Altogether, these findings demonstrate that inhibition of NF-κB, which results in downstream inhibition of YY1 and XIAP, results in inhibition of YY1 activity, leading to upregulation of DR5 and subsequent sensitization to TRAIL. The findings are in a manuscript that will soon be submitted for publication (Huerta-Yepez et al., 2006). The abstract is highlighted below.

Abstract

Most tumors are resistant to TRAIL and need to be sensitized to undergo apoptosis. We have recently reported that TRAIL-resistant human prostate carcinoma cell lines can be sensitized by various NF-κB inhibitors (Huerta-Yepez et al., 2004), and sensitization correlated with upregulation of DR5 expression. We hypothesized that a gene product(s) regulated by NF-κB with DR5 repressor activity may be responsible for the DR5 regulation. Inhibition of NF-κB activity resulted in significant upregulation of DR5 expression and sensitized prostate tumor cells to TRAIL-mediated apoptosis and synergy is achieved. Treatment of PC-3 cells with NO inhibited both NF-κB and YY1 DNA-binding activity and also inhibited YY1 expression. Treatment of PC-3 cells with YY1 siRNA resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The direct role of YY1 in the regulation of DR5 expression was examined in an DR5 luciferase reporter system (pDR5). Two constructs were generated, the pDR5/-605 construct with a deletion in the promoter region containing the putative YY1 DNA-binding region (-1224 to -605) and a construct pDR5-YY1 with a mutation of the YY1 DNA-binding site. Transfection of PC-3 cells with these two constructs resulted in

significant (3-fold) augmentation of luciferase activity over baseline suggesting the repressor activity of YY1. The present findings demonstrate that YY1 negatively regulates DR5 transcription and expression and hence, regulates resistance to TRAIL-induced apoptosis. Inhibitors of YY1 expression and/or activity in combination with TRAIL may be useful in the treatment of TRAIL-resistant tumor cells.

3. Chemical modification of the transcription repressor YY1 by nitric oxide: mechanism of NO-induced upregulation of DR5 and sensitization to TRAIL-induced apoptosis

The above findings in section 1 (above) and 3 (below) have clearly demonstrated that DR5 transcription is negatively regulated by the overexpression of the transcription repressor YY1 in CaP and overexpression of YY1 is regulated by the constitutively activated NFkB activity which also regulates XIAP. In addition to the transcription regulation of YY1 by NO donors and by drugs, we demonstrate that NO also directly modifies YY1 via S-nitrosylation and thus, preventing its DNA binding activity and transcription DR5 repressor activity. These studies have been recently published (Hongo et al., 2005) and the abstract is presented below.

Abstract

Treatment of several prostate cancer (CaP) cell lines (PC-3, CL-1, and DU-145) with the nitric oxide (NO) donor DETA/NONOate upregulated Fas expression and sensitized the CaP cells to the Fas ligand CH-11 agonist monoclonal antibody-induced apoptosis. Previous findings demonstrated that the transcription repressor Yin Yang 1 (YY1), which is inhibited by NO, negatively regulates Fas transcription [H.J. Garban, B. Bonavida, Nitric oxide inhibits the transcription repressor Yin-Yang 1 binding activity at the silencer region of the Fas promoter: a pivotal role for nitric oxide in the upregulation of Fas gene expression in human tumor cells, J. Immunol. 167 (2001) 75-81]. YY1 is a zinc finger protein and thus, we hypothesized that NO inhibits YY1 activity via S-nitrosation of critical cysteines residues coordinated by $Zn2+$. Treatment of PC-3 cells with DETA/NONOate inhibited the constitutive DNA-binding activity of YY1 as assessed by EMSA. Further, treatment with DETA/NONOate resulted in S-nitrosation of YY1 as detected by two different methods. The DAN-based method examined NO-treated tumor-derived cell lysates that were immunoprecipitated with an anti-YY1 specific antibody and the NO released was determined quantitatively by fluorometry. The second method consisted of immunoprecipitation of the tumor cell lysates by an anti-SNO cysteine antibody and the immunoprecipitate was immunoblotted with anti-YY1 antibody. Both methods revealed significant S-nitrosation of YY1 by DETA/NONOate treatment over control untreated cells. The S-nitrosation of YY1 was further corroborated by immunohistochemistry using dual color immunofluorescence. The direct role of YY1 in the negative regulation of Fas expression was demonstrated by transfection of cells with siRNA YY1. The transfectants exhibited upregulation of Fas expression in the absence of treatment with DETA/NONOate and were sensitized to CH-11-induced apoptosis. Altogether, these findings reveal that NO inhibits YY1 DNA-binding activity through S-nitrosation and consequently results in upregulation of Fas expression and tumor cell sensitization to Fas-induced apoptosis.

4. Role of chemotherapeutic drugs in the sensitization of CaP to TRAIL-induced apoptosis

Previous studies have reported that treatment of tumor cell lines with chemotherapeutic drugs such as adriamycin or CDDP resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The mechanism underlying the drug-induced regulation of DR5

expression is not known. Our studies with the chemotherapeutic drugs corroborate the above findings. In addition, we demonstrate that treatment of CaP with CDDP resulted in inhibition of NF-κB and YY1 activities, resulting in upregulation of DR5. We demonstrate that inhibitors of XIAP, NF-κB and YY1 mimic CDDP-induced effects. In addition, we demonstrate that YY1 is a transcription repressor and negatively regulates DR5 expression and thus, its inhibition by CDDP, reverses the repression and upregulates DR5 transcription and sensitizes CaP to TRAIL-induced apoptosis. Further studies, as demonstrated above for NO-mediated effects using reporter systems, confirmed the role of CDDP-induced inhibition of YY1 in the regulation of DR5 expression and sensitivity to TRAIL. A manuscript has been prepared and will soon be submitted for publication (Baritaki et al., 2006; Appendix). The abstract of this publication in highlighted below.

Abstract

Cancer patients initially respond to treatment with chemotherapy, however, recurrences occur and the tumors become refractory to further chemotherapy. Immunotherapy is currently being investigated as an alternative to overcome drug resistance. TRAIL, a member of the TNF family, has been shown to kill sensitive tumor cells with minimal toxicity to normal tissues and is a new candidate for immunotherapy. Many drug-resistant tumor cells are also resistant to TRAIL and such tumors require sensitization to reverse TRAIL resistance. We and others have reported that several sensitizing agents (ex. VP-16, CDDP, ADR, chemical inhibitors, etc.) in combination with TRAIL result in reversal of resistance to TRAIL apoptosis. Sensitization correlated with the upregulation of DR5 expression. This study examined the mechanism underlying the upregulation of DR5 expression. We hypothesize that the sensitizing agents may inhibit a transcription repressor acting at the DR5 promoter. Treatment of drug resistant PC-3 tumor cells with drugs (example CDDP, vp-16, adriamycin, vincristine) sensitized the tumor cells to TRAIL-induced apoptosis and apoptosis correlated with upregulation of DR5 expression and inhibition of YY1. By examining the promoter of DR5, we detected the presence of one putative binding site for the transcription repressor YY1. We examined whether YY1 negatively regulates DR5 transcription and whether YY1 inhibition by the drug upregulates DR5 expression. We used PC-3 cells transfected with a luciferase reporter system (pDR5 WT) and plasmids in which the YY1 binding site was either deleted (pDR5 -605) and/or mutated (pDR5/YY1 mutant). The findings revealed that the baseline reporter activity was significantly augmented in cells transfected with either the deleted or mutated plasmids. In addition, CDDP treatment augmented the luciferase activity in the WT reporter system, whereas there was no augmentation in the deleted or mutant transfected cells. The direct role of YY1 in the upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis was demonstrated in cells treated with siRNA YY1. The findings demonstrate that drug-induced upregulation of DR5 and sensitization to TRAIL is mediated through inhibition of the transcription repressor YY1. Inhibition of YY1 correlated with sensitization to TRAIL-induced apoptosis.

5. Role of XIAP and Bcl-$_{xL}$ expression in the regulation of PC-3 resistance to CDDP-induced apoptosis

We have found that prostate cancer tumor cell lines (PC-3, CL-1, LNCaP) are resistant to CDDP-mediated apoptosis. We examined whether the resistance is due in part to the constitutive activation of NF-κB and downstream regulation of XIAP and Bcl-$_{xL}$ expression similar to the resistance observed against TRAIL. We also hypothesized that tumor-derived cytokines (e.g.

TNF-a) that regulates the constitutive activity of NF-κB and downstream anti-apoptotic gene products like XIAP and Bcl-$_{xL}$ will result in the regulation of tumor cell resistance to CDDP. Hence, interfering with this pathway should sensitize the cells to CDDP apoptosis. This hypothesis was tested and verified experimentally. We have found that inhibition of endogenous TNF-α by recombinant sTNFR1 sensitizes PC-3 cells to CDDP-induced apoptosis. Further, inhibition of NF-κB by Bay 11-7085 mimicked the neutralization of TNF-α and sensitized the cells to CDDP apoptosis. The inhibition of NF-κB resulted in the inhibition of XIAP and Bcl-$_{xL}$. We demonstrate that inhibition of Bcl-$_{xL}$ by the inhibitor 2-methoxyantimycin A sensitizes cells to CDDP-induced apoptosis. The direct role of XIAP in the inhibition of CDDP-induced apoptosis was examined by the use of actinomycin D which we have earlier reported selectively inhibits XIAP expression (Ng and Bonavida, 2002). Treatment of PC-3 with Act D resulted in sensitization of PC-3 cells to CDDP-induced apoptosis. These findings demonstrate that NF-κB and gene products XIAP and Bcl-$_{xL}$ regulate the resistance of PC-3 cells to CDDP-induced apoptosis. These studies and others in progress will be comleted for publication. The above preliminary findings were presented at a mini-symposium in the 2004 AACR meeting in Orlando, Florida (Huerta-Yapez et al., 2004b)

6. Role of TNF-derived cytokine in the regulation of TRAIL-induced apoptosis.
   We have proposed to examine the role of TNF derived from prostate cancer cell lines in the regulation of TRAIL-induced apoptosis via autocrine/paracrine loop. This loop results in the activation of NF-κB and downstream upregulation of YY1 and XIAP. Studies were performed with the PC-3 cell line that secretes TNF-α and we demonstrate that TNF-α regulates tumor cell sensitivity to death receptor-induced apoptosis via NF-κB and YY1. The initial studies were performed examining the regulation of Fas and sensitivity to Fas as we already previously reported that YY1 regulates Fas. This study has been completed and is to be submitted (Huerta-Yepez *et al.,* 2006—abstract shown below). Similar preliminary studies have been performed and examining the regulation of TNF-α autocrine loop of the TRAIL receptor DR5 and similar findings to Fas were observed. These studies are currently being completed.

Abstract
   Tumor cells are invariably resistant to Fas ligand-induced apoptosis. This study examined the role of tumor-derived TNF-α (autocrine/paracrine loop) in the regulation of tumor-cell resistance to FasL-induced apoptosis. We have reported that Fas expression and sensitivity to FasL is negatively regulated by the transcription repressor factor Yin Yang 1 (YY1). We hypothesized that TNF-α-induces the activation of NF-κB and the transcription repressor Yin Yang 1 (YY1), both of which negatively regulate Fas expression and sensitivity to FasL-induced apoptosis. This hypothesis was tested in PC-3 prostate cancer cells which synthesize and secrete TNF-α and express constitutively active NF-κB and YY1. Treatment of PC-3 cells with TNF-α (10 units) resulted in increased NF-κB and YY1 DNA-binding activity, upregulation of YY1 expression, downregulation of surface and total Fas expression and induced-resistance of PC-3 to apoptosis induced by FasL agonist antibody CH-11. In contrast, blocking the binding of secreted TNF-α on PC-3 cell with soluble recombinant sTNF-RI resulted in significant inhibition of constitutive NF-κB and YY1 DNA-binding activity, downregulation of YY1 expression, upregulation of Fas expression and sensitization to CH-11-induced apoptosis. The regulation of YY1 expression and activity by NF-κB was demonstrated by the use of the NF-κB inhibitor

Bay11-7085 and by the use of a GFP reporter system whereby deletion of the YY1 tandem binding site in the promoter significantly enhanced GFP expression. The direct role of YY1 expression in the regulation of PC-3 resistance to CH-11-induced apoptosis was shown in cells transfected with siRNA YY1 whereby such cells exhibited upregulation of Fas expression and were sensitized to CH-11-induced apoptosis. These findings demonstrate that the TNF-α autocrine-paracrine loop is involved in the constitutive activation of NF-κB and YY1 in the tumor cells and hence leading to inhibition of Fas expression and resistance to Fas-induced apoptosis. These findings also reveal new targets such as TNF-α, NF-κB and YY1 whose inhibition can reverse tumor cell resistance to Fas-mediated apoptosis.

Other relevant publications supported by the DOD:

1. A review on the transcription repressor YY1 (Gordon et al., 2005).

We have published a review on the transcription repressor YY1, which was based, in part, on our findings of the role of this transcription repressor in both drug and immune-induced apoptosis in prostate cancer intensive cells. This review refers to the studies that we have published and that are to be published from the DOD grant. The abstract is highlighted below.

A*bstract*

The ubiquitous transcription factor Yin Yang 1 (YY1) is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation. YY1 exerts its effects on genes involved in these processes via its ability to initiate, activate, or repress transcription depending upon the context in which it binds. Mechanisms of action include direct activation or repression, indirect activation or repression via cofactor recruitment, or activation or repression by disruption of binding sites or conformational DNA changes. YY1 activity is regulated by transcription factors and cytoplasmic proteins that have been shown to abrogate or completely inhibit YY1-mediated activation or repression; however, these mechanisms have not yet been fully elucidated. Since expression and function of YY1 are known to be intimately associated with progression through phases of the cell cycle, the physiologic significance of YY1 activity has recently been applied to models of tumor biology. The majority of the data are consistent with the hypothesis that YY1 overexpression and/or activation is associated with unchecked cellular proliferation, resistance to apoptotic stimuli, tumorigenesis and metastatic potential. Studies involving hematopoetic tumors, epithelial-based tumors, endocrine organ malignancies, hepatocellular carcinoma, and retinoblastoma support this hypothesis. Molecular mechanisms that have been investigated include YY1-mediated downregulation of p53 activity, interference with poly-ADP-ribose polymerase, alteration in c-myc and nuclear factor-kappa B (NF-kappaB) expression, regulation of death genes and gene products, and differential YY1 binding in the presence of inflammatory mediators. Further, recent findings implicate YY1 in the regulation of tumor cell resistance to chemotherapeutics and immune-mediated apoptotic stimuli. Taken together, these findings provide strong support of the hypothesis that YY1, in addition to its regulatory roles in normal biologic processes, may possess the potential to act as an initiator of tumorigenesis and may thus serve as both a diagnostic and prognostic tumor marker; furthermore, it may provide an effective target for antitumor chemotherapy and/or immunotherapy.

2. A review on biomarkers in cancer tissues

We have presented our findings in a minisymposium on the role of various biomarkers including XIAP in cancers and their prognostic significance. The studies were published (Bonavida et al., 2005; Appendix). The abstract is highlighted below.

Abstract

PRIMARY OBJECTIVE: The primary objective is to delineate the potential utility of cancer biomarkers that correlate and predict response to immunotherapy in cancer patients who are refractory to conventional therapeutics. Unlike significant development of biomarkers that predict response to chemotherapy, very few biomarkers have been developed to predict the response to immunotherapy. MAIN OUTCOMES AND RESULTS: This article describes briefly the importance of characterizing and validating biomarkers for immunotherapy. A few examples have been provided, such as the transcription factor NF-kappaB, the transcription repressor Yin-Yang 1 (YY1), the pro-apoptotic gene product (Smac/DIABLO) and the circulating Fas and Fas ligand. These biomarkers have been determined to be of prognostic significance in different cancers. CONCLUSIONS: Immunotherapy is considered as an alternative therapy in the treatment of cancer patients who are refractory to chemotherapy/radiation/hormonal therapies. Cross-resistance to apoptosis develops between cancer cells that are resistant to conventional therapeutics and immunotherapy. Therefore, it is important to develop biomarkers that will determine patient response to immunotherapy.

3. A solicited review on tumor immunology (Bonavida, 2006).

4. Studies on the regulation of chemoresistance via NF-κB-induced upregulation of the anti-apoptotic gene product Bcl-$_{xL}$ in B-NHL cell lines.

These studies examined the mechanism by which rituximab (anti-CD20 antibody) sensitizes tumor cells to drug-induced apoptosis. We have found that there was selective inhibition of Bcl-$_{xL}$ expression which is under the transcriptional regulation of NF-κB and the AP-1 transcription factors. We have also examined the role of the IAP (Inhibitors of Apoptosis) family. These studies are related to the studies supported by the DOD on the regulation of resistance (Jazirehi et al., 2003; 2004; Appendix).

Personnel supported by this grant (12/15/01 to 12/14/05)

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Hongo, Fumiya
Jazirehi, Alireza
Neshat, Mehran S.

Key Research Accomplishments

The following are the key research accomplishments generated during the DOD grant period 2001-2005.

1) We have demonstrated that the overexpression of the anti-apoptotic gene product XIAP in prostate cancer cell lines is involved in the maintenance and resistance of the cell lines to TRAIL-induced apoptosis. The overexpression of XIAP was found in all cell lines, androgen-dependent and androgen-independent.

2) We have demonstrated that overexpression of XIAP and resistance to TRAIL-induced apoptosis was due in part to the low expression of Smac/DIABLO. Following treatment of the cell lines with TRAIL, there was activation of the mitochondria and release of lower level Smac/DIABLO which was not effective in counteracting the inhibitory activity of overexpressed XIAP and thus, no activation of caspases 9, 7 and 3. We demonstrate that transfection of cells with Smac/DIABLO to upregulate its cytotoxic level resulted in activation of caspases 9 and 3 and reversing resistance to TRAIL-induced apoptosis.

3) We have shown that inhibition of the constitutively activated NF-κB in prostate cancer cell lines resulted in inhibition of XIAP expression and also sensitized the cells to TRAIL-induced apoptosis. We demonstrate that NF-κB can be inhibited following treatment with chemical inhibitors such as Bay 11-7085 and DHMEQ, the nitric oxide donor DETANONOate and also following treatment with chemotherapeutic drugs such as CDDP and all these inhibitors inhibit XIAP and sensitize cells to TRAIL-induced apoptosis.

4) Two complementary signal models were proposed for TRAIL-induced apoptosis in TRAIL resistant tumors: Signal 1 is involved in the inhibition of XIAP expression (i.e. Smac/DIABLO transfection/drugs like Actinomycin D) and signal 2 provided by TRAIL triggers the mitochondrial pathway for apoptosis.

5) We have shown that the regulation of resistance to TRAIL by NF-κB and XIAP was due, in large part, to the negative regulation of transcription of the TRAIL death receptor DR5. Hence, inhibition of NF-κB resulted in upregulation of DR5 and sensitization to TRAIL-induced apoptosis.

6) We have examined the mechanism by which NF-κB negatively regulates DR5 expression and demonstrated that the inhibition is indirect and is primarily mediated via the transcription repressor Yin Yang 1 (YY1) that is regulated upstream by NF-κB.

7) We demonstrate the direct role of YY1 in the negative regulation of DR5 and the resistance to TRAIL-induced apoptosis by various means: A) We demonstrate that treatment with the NO donor DETANONOate inhibits YY1 expression and DNA-binding activity via S-nitrosylation of YY1. B) We demonstrate that transfection of cells with siRNA for YY1 resulted in upregulation of DR5 and sensitivity to TRAIL. C) We demonstrate using a DR5 reporter system that deletion of the region containing the putative YY1 DNA-binding site or mutation of the YY1 binding site resulted in significant upregulation of DR5 luciferase activity.

8) We have examined the mechanism by which the prostate cancer cell lines have constitutively activated NF-κB and overexpression of YY1. We demonstrate in the PC-3 cell line, which secretes TNF-α, that TNF-α via an autocrine/paracrine loop is responsible in large part in the activation of NF-κB, XIAP, and YY1. Hence, blocking of the loop resulted in inhibition of these gene products and upregulation of DR5 and sensitivity to TRAIL.

9) The role of NF-κB, XIAP and YY1 in the regulation of TRAIL resistance was corroborated using specific inhibitors of the NF-κB pathway. Hence, treatment with Bay 11-7085 or DHMEQ resulted in inhibition of YY1, inhibition of NF-κB and XIAP, upregulation of DR5 and sensitivity to TRAIL-induced apoptosis.

10) We have found that constitutive activation of NF-κB and the overexpression of YY1 in the cytosol and the nucleus, by IHC analysis in prostate cancer tissue microarray is of prognostic significance.

11) We have also shown that overexpression of XIAP regulates chemotherapeutic drug-induced apoptosis, and inhibition of XIAP results in the activation of the apoptotic pathway mediated by the chemotherapeutic drug.

12) We have examined the clinical significance of XIAP overexpression in prostate cancer. We have analyzed prostate cancer tissue microarrays and demonstrated by immunohistochemistry that XIAP overexpression is of prognostic significance.

13) We have also found that NF-κB regulates Bcl-$_{xL}$ expression in PC-3 cells. Inhibition of Bcl-$_{xL}$ activity results in sensitization of the cells to TRAIL-mediated apoptosis. These findings reveal that in PC-3, both XIAP and Bcl-$_{xL}$ overexpression regulates the sensitivity of prostate cancer cells to TRAIL apoptosis.

Reportable Outcomes

<u>Publications</u>

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Conclusions

Our studies in prostate cancer cell lines have demonstrated that the anti-apoptotic gene product XIAP is directly involved in the regulation of TRAIL-induced apoptosis and also in drug-induced apoptosis. Since XIAP transcription is regulated in large part by NF-κB and NF-κB is constitutively activated in prostate cancer cells, the overexpression of XIAP is of significant clinical importance. It is a target for therapeutic intervention to reverse immune resistance to various immuno-therapeutic interventions. In addition, XIAP is also a target for chemotherapeutic intervention in highly refractory tumor cells. Our studies have implicated several means by which one can overcome the resistance of the prostate cancer cells to TRAIL-induced apoptosis. These include interference with growth factors such as TNF-α that are secreted by the tumor cell and/or present in the tumor micro-environment that regulate the transcription activation of NF-κB and in turn regulates the expression of XIAP and maintains the tumor cells resistant to various therapeutics. Thus, it is possible to interfere with inhibition of the growth factor to inhibit the loop and restore sensitivity of tumor cells through therapeutics. In addition, we have identified several intracellular targets that can regulate the sensitivity of the cells to TRAIL-induced apoptosis. In addition to inhibitors of the NF-κB pathway, we have also identified a transcription repressor factor, Yin Yang 1 (YY1), which is under the transcription regulation of NF-κB and is directly involved in the negative regulation of DR5 transcription and resistance to TRAIL-induced apoptosis. This transcription factor has been reported by us to be overexpressed in human prostate cancer tissues and of prognostic significance. The identification of YY1 in the regulation of TRAIL sensitivity provides us with a new target for intervention to restore sensitivity. This was corroborated in studies demonstrating that inhibitors of YY1, such as nitric oxide donors, siRNA and inhibitors of NNF–κB all resulted in inhibition of this transcription repressor and sensitization to TRAIL. In addition, we have demonstrated that the DR5 promoter has a YY1 DNA-binding site whose deletion or mutation upregulates DR5 and restores sensitivity to TRAIL. Since current studies are exploring the therapeutic potential of TRAIL and/or agonist antibodies against DR4 or DR5, our findings are directly relevant in that they provide novel approaches to enhance the therapeutic efficacy of TRAIL or agonist antibody in patients who do not respond or respond poorly to such treatments alone. This is also true for treatment with chemotherapeutic drugs. Thus, our findings suggest that combination treatment with inhibitors for YY1 in combination with TRAIL or antibody could overcome TRAIL and drug resistance. Our findings showing that TRAIL sensitivity is under the control of NF-kB, XIAP and YY1 suggests that these factors are therapeutic targets. Currently, various inhibitors of NF-kB are being tested clinically and these may be applicable in combination with TRAIL in TRAIL-resistant tumors. In addition, there have been reports of the use of small molecule antagonists for XIAP which show significant pre-clinical anti-tumor effects when used alone or in combination with chemotherapeutic drugs or TRAIL. We also show that inhibitors of YY1 such as nitric oxide donors or siRNA can restore sensitivity to TRAIL-induced apoptosis and such inhibitors have potential clinical relevance.

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Appendices

Please see the following pages.

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A New Challenge for Successful Immunotherapy by Tumors That Are Resistant to Apoptosis: Two Complementary Signals to Overcome Cross-Resistance

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Tumor resistance to conventional therapies is a major problem in cancer treatment. While tumors initially respond to radiation or chemotherapies, subsequent treatments with these conventional modalities are ineffective against relapsed tumors. The problem of tumor resistance to chemotherapy and radiation has led to the development of immunotherapy and gene-based therapies. These alternative therapeutic approaches are intensely explored because they are supposed to be more tumor specific and better tolerated than the conventional therapies. Recent advances in apoptosis have revealed that resistance to apoptosis is one of the major mechanisms of tumor resistance to conventional therapies. Resistance to apoptosis is a naturally acquired characteristic during oncogenesis and is selected for after successive rounds of conventional therapies. Resistance to apoptosis involves dysregulation and/or mutation of apoptotic signaling molecules that render tumor cells unresponsive to apoptotic stimuli. Since both

immunotherapy and chemotherapy kill tumors by apoptosis and the killings are signaled through a central core apoptotic program, dysregulation of this central program and development of resistance to apoptosis in chemoresistant cells could render them cross-resistant to immunotherapy. Therefore, in order to establish an effective antitumor response and to complement immunotherapy and gene-based therapies, cross-resistance due to resistance to apoptosis must be overcome. In this review, based on prior findings and recent evidence, we put forth a model, verified experimentally, in which chemoresistant tumor cells can be sensitized to immune-mediated killing by subtoxic concentrations of chemotherapeutic drugs/factors. The model involves two complementary signals. The first signal is a sensitizing signal that regulates pro/antiapoptotic targets, thus facilitating the apoptotic signal. The second apoptotic signal initiates a partial activation of the apoptotic signaling pathway, and activation is completed by complementation with signal one. Thus, effective killing of immunoresistant cells is achieved by both signals. The two-signal approach provides a new strategy to overcome cancer cross-resistance to immunotherapy and opens new avenues for the development of more effective and selective immunosensitizing agents. © 2002, Elsevier Science (USA).

I. INTRODUCTION

Significant advances have been made in the treatment of cancer by chemotherapeutic drugs. However, the development and/or acquisition of tumor resistance to chemotherapy presents a major problem (Patel and Rothenberg, 1994). While patients with early and localized tumors respond to standard chemotherapeutic treatments, the majority of cancer patients afflicted with advanced metastatic tumors are unresponsive to further chemotherapeutic treatments. These patients will eventually succumb to incurable disease due to relapse of drug-resistant tumors. The failure to eradicate resistant tumors with standard chemotherapeutic treatments calls for the use of more aggressive therapeutic regimens in both higher dosage and longer duration, but these attempts only exacerbate systemic toxicity and immunosuppression while having little effect on tumor cure and survival (MacNeil and Eisenhauer, 1999). Another problem with the prolonged use of chemotherapeutic agents for cancer treatment is the selection for increasingly drug-resistant tumor clones that may actually accelerate tumor progression toward the incurable status (Schulze and Isaacs, 1990). Thus, a detailed understanding of the molecular mechanisms of tumor drug resistance is critical for designing strategies to overcome the problem of resistance and for improving the therapeutic outcome.

The mechanism of drug resistance is complex. In solid epithelial tumors, external factors such as poor vascular access and little drug penetration into the tumor mass are thought to be involved in the development of resistance (Simpson-Herren and Noker, 1991). Other proposed mechanisms are decreased cellular drug uptake or increased efflux, metabolic inactivation of

drugs, detoxification of drug-associated toxic metabolites, enhanced DNA repair mechanisms, and amplification or compensation of drug target genes (reviewed in Stavrovskaya, 2000). Some of these mechanisms are frequently associated with the phenotype of multidrug resistance (MDR), which describes the cross-resistance to multiple chemically unrelated substances (Stavrovskaya, 2000; Biedler and Riehm, 1970). Extensive evidence has illustrated that P-glycoprotein, a drug efflux pump, is a major contributor to the MDR phenotype in various cancers (Gottesman and Pastan, 1993). Consequently, much of the research effort today has focused on searching for alternative therapeutic strategies that aim to reverse or bypass these drug-related resistance mechanisms (Tan et al., 2000).

Failure to cure chemoresistant tumors with traditional chemotherapeutic approaches has led to the introduction of immunotherapy. In practice, tumor immunotherapy is an ideal therapeutic approach because it offers several advantages over chemotherapy including low organ toxicity and high tumor selectivity. In immunotherapy, the tumor-killing agents are derived from the host's own immune system such as lymphokine-activated killer cells (LAK) and interleukin-2 (IL-2)-activated tumor-infiltrating lymphocytes (TIL) (Kurnick and Kradin, 1991). Thus, these approaches are considered to be better tolerated and can induce less undesirable organ toxicity than chemotherapy (Kurnick and Kradin, 1991). Also, based on the principle that most tumors have the capacity to trigger an immune response, immunotherapy can be used for the selective and specific recognition of tumor targets by the generation of specific antitumor cytotoxic T cell responses (Sogn, 1998).

Immunotherapeutic strategies under investigation consider that chemoresistant tumors are sensitive to immunotherapy, and it has been assumed that immunotherapy attacks tumor cells using different mechanisms of action and may not be subjected to the mechanisms of drug resistance discussed previously. Despite these proposed advantages over chemotherapy, immunotherapy today still fails to deliver a significant curative rate and largely remains an experimental therapeutic approach. Initially, immunotherapy, or T-cell based immunotherapeutic approaches (LAK and TIL), have generated a great deal of excitement when they were shown to be effective in certain transplanted tumor models in mice (Mule et al., 1984). The initial experiments in these tumor models proved, in principle, that activated cytotoxic T cells (CTLs) and natural killer (NK) cells in both LAK and TIL systems can recognize tumor cells and elicit a potent antitumor killing. However, subsequent studies with the LAK and TIL systems in clinical trials with patients failed to demonstrate a significant response rate (Rosenberg et al., 1987). Further studies with cytokine gene transfer into tumor targets and pulsing CTLs with specific tumor peptides (i.e., tumor vaccines) still proved to be unsuccessful in long-term cures as well (Tan et al., 1996; Cormier et al., 1997; Salgaller et al., 1996). Clearly, T-cell based immunotherapy has its own limitations.

Even though considerable progress has since been made on the identification of specific tumor antigens, and numerous elaborate immunological manipulations have been invented for raising a strong specific antitumor response, spontaneous drug-resistant tumors remain virtually resistant to immunotherapy in most patients (Sogn, 1998). The lack of a significant positive response by immunotherapy against the drug-resistant tumor cells suggests that the mere manipulation of the immune system may not be sufficient to restore a positive antitumor killing. Other aspects such as tumor sensitivity to killing by cytotoxic T cells must also be considered.

While the original impetus for exploring immunotherapy as a potential cancer therapy is to overcome tumor resistance to chemotherapeutic drugs, it is unclear if drug-resistant tumors are actually sensitive to killing mediated by CTLs. One possibility that may explain the poor effectiveness of immunotherapy is that although immune cells could recognize chemoresistant tumors, chemoresistant tumors are also equally resistant to immune-mediated killing mechanisms. If tumors are cross-resistant to immune attacks, then further development on the technologies to improve immune recognition of tumor targets will not be of significant benefit. Thus, the success of immunotherapy will ultimately be dictated by both the presence of antitumor CTLs and the sensitivity of tumor targets to the killing mediated by these cells. In addition, tumor chemoresistance may actually reflect a part of the general tumor resistance mechanism to a common cytotoxic pathway mediated by various different stimuli, namely apoptosis or programmed cell death, and such a resistance scheme to a central cytotoxic pathway may also render the cells resistant to immune-mediated killing (Reed, 1999). The definition of cross-resistance actually could go beyond the multidrug resistance phenomenon and encompass other cytotoxic stimuli, including the immune cells. Therefore, the ultimate goal for a successful antitumor therapy, be it chemotherapy or immunotherapy, is to overcome cross-resistance for the induction of apoptosis. Many physiological and external stimuli can induce apoptosis in susceptible tumor cells, including both chemotherapeutic drugs and host-activated immune cells (Reed, 1999). However, not all tumor cells are intrinsically sensitive to apoptosis. Most advanced malignant tumor cells develop resistance to apoptosis by negatively regulating the apoptotic pathways that are triggered by chemotherapeutic drugs or activated immune cells (Reed, 1999).

With the premise that chemoresistant tumors develop general mechanisms of resistance to apoptosis-mediated stimuli, the hypothesis put forth here for an effective antitumor therapeutic strategy is to utilize complementary proapoptotic signals to overcome tumor resistance to immune-mediated apoptosis. The following discussions summarize the current experimental approaches demonstrated by others and by us for sensitization of resistant tumor cells to immune-mediated apoptosis. This review also describes the

molecular mechanisms of cross-resistance to chemotherapeutic drugs and immunocytotoxics and how these approaches of immunosensitization selectively influence the immune-mediated apoptotic signaling pathway.

II. APOPTOSIS AS CYTOTOXIC MECHANISMS OF T LYMPHOCYTES

Apoptosis, or programmed cell death (PCD), plays an important role in the induction of tumor cell death (Martin and Green, 1994). It is a genetically programmed cell suicide process that also plays a pivotal role in normal development and physiology (Meier *et al.*, 2000). Dysregulation of this process may lead to pathological states such as cancer and autoimmune diseases (Thompson, 1995). The final phenotypic features of apoptosis include membrane blebbing, chromatin condensation, and DNA fragmentation (Wyllie *et al.*, 1980). The molecular machinery for executing apoptosis is inherently in place in most cells, including tumor cells, but it is only triggered with the proper stimulus.

There are two major cytotoxic mechanisms by which activated T lymphocytes induce apoptosis: the granule-exocytosis pathway, mediated primarily by perforin and granzymes, and the death receptor signaling pathways, which involve the apoptosis-inducing tumor necrosis factor (TNF)-related ligand family protein members (Fas/CD95, TNFα, and Apo2L/TRAIL) (reviewed in Shresta *et al.*, 1998). The induction of the granule-exocytosis pathway requires direct lymphocyte to tumor cell contact and TCR/major histocompatibility complex (MHC) engagement and recognition in order to initiate the release of cytotoxic granules containing perforin, granzymes, and other cytotoxic constituents. The release of perforin results in polymerization of perforin that forms pores on the target cell surface and allows the passage of granzymes from the lymphocyte to the target cell interior. The granzymes trigger apoptosis by either activating a caspasedependent central apoptotic program or cleaving directly some of the substrates that are also cleaved by caspases. Perforin, however, induces necrotic cell death by causing cell membrane damage and releasing intracellular contents.

The TNF apoptosis-inducing ligand members trigger death receptor-mediated apoptosis in tumor target cells upon crosslinking with these death receptors on the cell surface (reviewed in Ashkenazi and Dixit, 1998). These cytotoxic TNF ligand members include TNFα, Fas/CD95, Apo2L/TRAIL, and Apo3L/Tweak. They can induce apoptosis in target cells either in the appropriate aggregated soluble form following secretion by the activated CTLs or in the membrane bound form on the surface of the activated lymphocytes. The role of the TNF-family ligand shed from lymphocytes and the activity of

soluble ligands have been addressed in a few reports. For instance, Tanaka *et al.* (1998) examined the role of soluble FasL compared to membrane bound FasL. They found that the membrane bound FasL was cytotoxic against target cells, whereas the sFasL was not. In fact, sFasL inhibited the cytotoxic activity of membrane bound FasL. Under certain conditions, however, the upregulation of Fas expression on target cells renders these cells susceptible to killing by sFasL. Tanaka *et al.* (1998) suggested that the membrane bound FasL are the physiological functional forms that induce apoptosis. Contrary to the granule-exocytosis system, the death receptor-mediated pathway is not restricted to TCR/MHC recognition provided that ligands expressed on the surface of the cytotoxic cells and corresponding receptors are expressed on the target cells. However, FasL expression on CTL is induced following activation by CTL-targeted cell recognition in an MHC restricted fashion. In this case, cell–cell contact is necessary. In addition, cell–cell contact may not be necessary if one uses recombinant soluble death ligand molecules for killing of sensitive target tumor cells (Shresta *et al.*, 1998).

Although the granule-exocytosis pathway was originally considered the primary killing mechanism against tumor cells, a number of studies suggest that death-receptor mediated apoptosis is just as, if not more, important for tumor killing by activated T lymphocytes (Frost *et al.*, 2001; Thomas and Hersey, 1998; Simon *et al.*, 2000; Lowin *et al.*, 1994). In studies utilizing CTLs deficient in perforin, killing could still be achieved with Fas/CD95-mediated components (Liu *et al.*, 1995). In addition, in the graft-verus-host disease model, death receptor-mediated apoptosis accounts for the majority of cytotoxicity (more than 85%) for CD4 + CTLs when matched with non-self MHC (Shresta *et al.*, 1998). Some of this death receptor-mediated killing could be attributed to non-MHC-restricted apoptosis induced by Fas/CD95 and Apo2L/TRAIL (Shresta *et al.*, 1998). Functional death receptor-mediated apoptosis is particularly important in immunotherapies utilizing CTLs restricted by non-self MHC such as the adoptive immunotherapy with allo-MHC-restricted CTLs in bone marrow-transplanted leukemia patients (Dazzi and Goldman, 1998; Stauss, 1999). Thus, a detailed study on the regulation of death receptor-mediated apoptotic pathway will improve the efficacy of current adoptive immunotherapy approaches and the use of recombinant ligands that can replace the cytotoxic lymphocytes.

III. THE ROAD MAP OF APOPTOSIS: ALL ROADS LEAD TO CASPASES

Recent advances in apoptosis have revealed two major apoptotic signaling pathways. One is initiated from the death receptors; the other originates from

the release of cytochrome c from the mitochondria (reviewed in Hengartner, 2000). Central to both apoptotic pathways is the activation of caspases, which is required for induction of the final apoptotic phenotype. To execute the final cellular demise, active caspases cleave and inactivate many key cellular proteins that maintain DNA integrity, cellular structural support, and metabolic functions. While both pathways converge to the defining feature of apoptosis—the activation cascade of effector caspases—the pathways are distinguished by their mechanisms of signal initiation.

A. The Death Receptor-Mediated Apoptosis Pathway

Members of the TNF death receptor superfamily (TNFRI, Fas/CD95/Apo-1, DR4/TRAIL-R1, DR5/TRAIL-R2, DR3/TRAMP) initiate the death receptor pathway, also termed the type I pathway (Reviewed in Ashkenazi and Dixit, 1998; Scaffidi *et al.*, 1998) (Fig. 1). Upon ligand receptor binding, the death receptors are clustered together into aggregation of trimers to form a death-inducing signaling complex (DISC) (Huang *et al.*, 1999). The induction of death signal by DISC requires the association of an adaptor protein FADD (Fas-associated death domain) with the cytoplasmic region of trimerized death receptors via interactions with their respective death domains (DD). FADD further recruits inactive zymogen forms of initiator caspases such as pro-caspase-8 or -10 through the association of its death effector domain (DED) with the N-terminal pro-domain region of the pro-caspases. The placement of the initiator pro-caspases in close proximity by the DISC induces the autocleavage and activation of the initiator pro-caspases. Cleaved subunits of pro-caspases assemble and become a unit of active holoenzyme that further activates downstream caspases by proteolysis. Active initiator caspases directly activate effector caspases pro-caspase-3 and initiate a proteolytic cascade of effector caspases (caspase-3, -6, and -7). Active effector caspases then digest key cytoplasmic and nuclear substrates that maintain cellular integrity, and lead to the final apoptotic features such as DNA fragmentation and membrane blebbing.

B. The Mitochondrial Apoptosis Pathway

The mitochondrial apoptosis pathway is initiated from the mitochondria, and it responds to both extracellular cues as well as internal insults such as DNA damage and high levels of intracellular reactive oxygen species (ROS) (reviewed in Green and Reed, 1998) (Fig. 1). The mitochondrial pathway is also termed as the "Type II" pathway (originally described in Scaffidi *et al.*, 1998). The pathway begins with the release of cytochrome c and
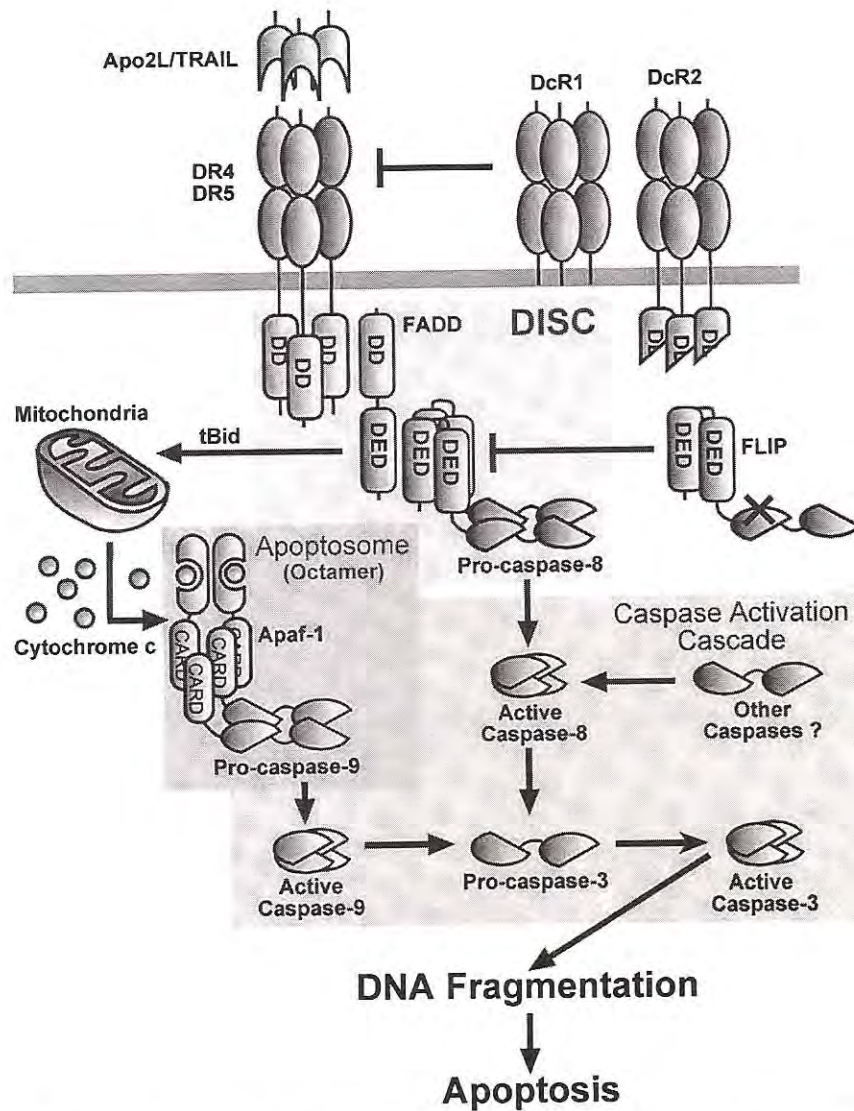
Fig. 1 Death receptor-mediated apoptosis pathway. Upon activation of DR4 and DR5, pro-caspase-8 is recruited via interactions with FADD. Decoy receptors DcR1 and DcR2 can inhibit the signal by competitive binding with TRAIL. Once pro-caspase-8 is recruited, it is auto-cleaved and activated. Active caspase-8 further activates downstream effector pro-caspases (pro-caspase-3 or other pro-caspases). Active caspase-3 then causes PARR cleavage and DNA fragmentation. An alternative mitochondrial pathway is possibly activated. When the mitochondrial pathway is activated, cytochrome c is released and binds to Apaf-1 to activate pro-caspase-9. Active caspase-9 can activate pro-caspase-3 and leads to DNA fragmentation and apoptosis.

Smac/DIABLO from the mitochondria into the cytoplasm, in response to mitochondrial membrane instability caused either by membrane pore formations with pro-apoptotic members of the Bcl-2 family or by disruption of the mitochondrial membrane potential. Cytochrome c serves as the apoptosis inducer, whereas Smac/DIABLO neutralizes the inhibition on the activation of caspase-9 (Ekert *et al.*, 2001). The role of Smac/DIABLO in death receptor-induced apoptosis was examined by us in TRAIL-resistant prostate cancer (Ng *et al.*, 1998). The presence of cytochrome c in the cytoplasm leads to the formation of an intracellular death-inducing complex named apoptosome. An apoptosome is a protein complex consisting of cytochrome c, Apaf-1 (apoptotic protease activator factor-1), pro-caspase-9, and possibly other regulatory proteins. Cytochrome c induces the oligomerization of Apaf-1, which undergoes an ATP-dependent conformational change and exposes its CARD domain (caspase recruitment domain) for the binding to pro-caspase-9, another cytosolic initiator caspase. Recruitment of pro-caspase-9 to the apoptosome leads to activation of pro-caspase-9 by a conformational change. The apoptosome containing caspase-9 activity is perceived to be an initiator holoenzyme, like active caspase-8, which further activates downstream effector caspases such as caspase-3 that leads to the full manifestation of the apoptotic phenotype.

It is important to note that the role of the mitochondrial pathway and Bcl-2 and homologues in Fas-mediated apoptosis is controversial. Studies reported that Bcl-2 and Bcl-xL can inhibit anti-Fas monoclonal antibody-induced apoptosis in some (type II) but not other lymphoid cell lines (type I) (Scaffidi *et al.*, 1998). However, studies by Huang *et al.* (1999) question the role of type II Fas-mediated apoptosis. They demonstrate that the findings with anti-Fas monoclonal antibody do not coincide with the physiological FasL on cytotoxic cells. They demonstrate clearly that Bcl-2 and Bcl-xL expressing targets can be killed by membrane bound or aggregated FasL. Further, the type II cells expressing or not expressing Bcl-2 or Bcl-xL were susceptible to cross-linked Fas monoclonal antibody. Thus, these findings demonstrate that the role of Bcl-2 is dependent on the condition as well as the nature of the cytotoxic ligand used in the studies.

IV. CROSS-TALKING BETWEEN THE TWO APOPTOTIC PATHWAYS AND CROSS-RESISTANCE

There has been some controversy regarding the role of the mitochondrial pathway in death receptor-mediated signaling or apoptosis. In the Fas signaling pathway for instance, in cells of the hematopoietic system, such as B and T cells, Fas-mediated apoptosis is independent of the expression of Bcl-2

(Strasser *et al.*, 1995). For such cell lines and lymphocytes, one might expect the absence of cross-talk between the death receptor and intrinsic apoptosis signaling pathways. However, under certain circumstances and depending on the target cells used, cross-talk can take place.

As divergent as these two pathways are, the death receptor pathway can actually divert its signal through the mitochondrial pathway in some cases. In certain tumor cells, termed the type I cells, the engagement of the death receptors with the TNF-related death-inducing ligands induces high level of active caspase-8 that is sufficient for the direct activation of caspase-3 (Hengartner, 2000; Scaffidi *et al.*, 1998). In these cells, the rate of caspase activation is rapid (4–6 hr) and does not involve mitochondrial signaling components. In type II cells, a minimal amount of caspase-8 is activated and the level is not sufficient to fully activate a caspase-3 enzymatic cascade (Scaffidi *et al.*, 1998). In this case, a low level of caspase-8 can divert its signal toward the mitochondrial pathway by cleaving the Bcl-2 family member BID (Luo *et al.*, 1998). The truncated BID then interacts with Bax to facilitate cytochrome c release from the mitochondria, inducing the formation of apoptosome and activation of caspase-9. The cross-talking between these two apoptotic pathways is important for sensitization to apoptosis, because even if the direct death receptor-mediated apoptotic pathway is blocked, there is still a possibility whereby the initial trigger can be utilized to kill tumor cells by diverting the signal to the mitochondrial pathway.

V. INHIBITION OF APOPTOSIS AS A MECHANISM OF CROSS-RESISTANCE

To ensure survival, organisms must protect themselves from external and internal insults by triggering protective programs that repair DNA or other cellular damages. However, when the damage is irreversible or too overwhelming for the repair mechanism, then the organism must eliminate the damaged cells by apoptosis, or programmed cell death. Failure to induce apoptosis in damaged cells in conjunction with the inability to control DNA damage promotes the generation of cancer (Reed, 1999). Thus, almost all cancer cells possess an inherent resistance scheme to apoptosis that permits accumulation of genetic mutations and further progression and expansion of highly malignant and invasive tumors. In normal cells, most protooncogenes promote apoptosis as well as cell proliferation; but in cancer cells, proapoptotic effects of protooncogenes are countered by oncogenes that inhibit apoptosis, thereby promoting the survival and proliferation effects of the oncogenes (Evan and Littlewood, 1998).

Strasser *et al.* (1990) have reported the generation of Bcl-2 and myc doubly transgenic mice. These mice develop tumors faster than the Eμ-myc mice. These findings establish the oncogenic role of Bcl-2 and its cooperation with myc. Studies by Davidson *et al.* (1998) show defective Fas-FasL interactions in *lpr* and *gld* lead to accumulation of massive numbers of lymphocytes. The findings reveal that by 1 year of age, 28% of C3H-*gld* mice and 57% of BALB-*gld* mice had monoclonal outgrowth of B cells in spleen and lymph nodes. After transfer into scid recipients, the majority of these clonal B cell populations gave rise to metastatic plasmacytoid tumors. Thus, normal Fas–FasL interactions prevent the development of B cell neoplasms. The risk of B cell lymphomagenesis associated with Fas and FasL mutations is not restricted to mice. Two out of four members with genomic Fas mutation and autoimmune lymphoproliferative disease were diagnosed with B cell lymphomas at 25 years of age (Lin *et al.*, 1995). In patients with multiple myelomas, Fas mutations were detected in 10% of tumors containing bone marrow expirates (Landowski *et al.*, 1997b). It is not clear, however, whether these tumors derive because of Fas mutation or whether an increased risk of neoplasm is due to cytotoxicity treatment and infection. Clearly, inhibition of apoptosis is crucial for the development of cancer.

Many cancer cell types are initially sensitive to drug/hormone mediated apoptosis. However, resistant variants and relapses result in cancer cells that are resistant to apoptosis induced by internal cellular cues. Such tumors also become cross-resistant to chemotherapy and immunotherapy. Indeed, numerous reports have documented that resistance to chemotherapeutic drugs and immunocytotoxics are related since they induce apoptosis using a similar caspase-dependent pathway (Los *et al.*, 1997; Landowski *et al.*, 1997a, 1999; Ding *et al.*, 2000; Wang *et al.*, 2000). A hierarchical pattern of tumor resistance to various apoptotic stimuli was documented previously in various cell lines that shows that chemoresistant cells are equally resistant to recombinant TNFα, whereas TNFα-resistant cells may not be resistant to chemotherapeutic drugs (Safrit and Bonavida, 1992). Similar findings were confirmed in a series of drug or immune selection experiments using multiple myeloma (MM) cells (Landowski *et al.*, 1997a, 1999). When the MM cells were selected for drug resistance, they also became resistant to Fas-mediated apoptosis. However, when they were selected for Fas resistance, they were not cross-resistant to drugs (Landowski *et al.*, 1997a, 1999). Further molecular analysis to elucidate the molecular basis of cross-resistance revealed that the deficiency of caspase-3 activation and inability to induce mitochondrial apoptotic events are associated with the drug/immune cross-resistant phenotype (Ding *et al.*, 2000). Interestingly, the MDR phenotype is also correlated with the reduction of caspase-3 activation and enhanced expression of anti-apoptotic molecules, suggesting that the MDR resistance mechanism is not

drug-specific and is likely to confer immunoresistance as well (Ding *et al.*, 2000).

VI. SENSITIZATION OF RESISTANT TUMOR CELLS TO CYTOTOXIC LYMPHOCYTES/FACTORS-MEDIATED APOPTOSIS

Since inhibition of apoptosis has been identified as the major resistance mechanism to tumor cell killing mediated by chemotherapeutic drugs, it is possible that modifications of the death receptor-mediated signaling pathway for apoptosis are affected. Thus, under these circumstances, death receptor-mediated signals by cytotoxic immune cells will not result in apoptosis of the tumor cells. Therefore, the modulation of the apoptotic pathways to reverse this resistance presents a unique opportunity to improve the outcome of current anticancer treatment strategies. Even though immunotherapy has not been successful in killing tumor cells due to the profound tumor immunoresistance to apoptosis, the immunosensitizing agents that modulate signaling molecules involved in the immune-mediated apoptosis may enhance tumor killing and improve the efficacy of immunotherapy. Currently, several lines of evidence have shown that subtoxic levels of genotoxic chemotherapeutic drugs (such as etoposide, cisplatinum, adriamycin, actinomycin D, camptothecin, and 5-fluorouracil) could serve as good immunosensitizing agents that can be used in combination with FasL, TNFα, or TRAIL to synergistically induce apoptosis in resistant tumor cells (Kinoshita *et al.*, 2000; Leverkus *et al.*, 2000; Mizutani *et al.*, 1999a,b, 2001; Mori *et al.*, 1999; Nagane *et al.*, 2000; Wen *et al.*, 2000). Similar sensitizing effects by subtoxic levels of drugs were also demonstrated in tumor target cells coincubated with LAK and TIL cells, as well as tumor specific activated T cells pulsed with tumor peptides (Frost *et al.*, 1997, 2001). Most importantly, the combination of drugs and TRAIL has been shown to act synergistically both *in vitro* and *in vivo* in tumor rejection, confirming that the approach achieves a better killing than high dose therapy while conferring little toxicity (Ashkenazi *et al.*, 1999).

A. Chemotherapeutic Drugs as Immunosensitizing Agents

Even though most advanced malignant tumor cells are resistant to chemotherapeutic drugs and immune attack, in many instances low levels of the same drugs could sensitize resistant tumor cells to immune-mediated

apoptosis (Kinoshita *et al.*, 2000; Leverkus *et al.*, 2000; Mizutani *et al.*, 1999a,b, 2001; Mori *et al.*, 1999; Nagane *et al.*, 2000; Wen *et al.*, 2000). This observation suggests that the sensitizing property of chemotherapeutic drugs is distinct from their direct apoptosis-inducing effects. Such a sensitizing effect has been documented even in cells that express the drug efflux pump, P-glycoprotein (Jazirehi *et al.*, 2001). Apparently, the effective intracellular concentration required for drug-mediated sensitization is much lower than what is needed to directly induce apoptosis. Several proposed molecular mechanisms of immunosensitization by chemotherapeutic drugs have been presented, including transcriptional upregulation of proapoptotic proteins and downregulation of antiapoptotic proteins (Fulda *et al.*, 2000; Wu *et al.*, 2000). The protein expression of signaling molecules and regulatory proteins involved in both the receptor-mediated pathway and the mitochondrial pathway and from the receptor to the effector caspase level are possibly subjected to modulations mediated by drugs (Fig. 2). Other possible mechanisms for immunosensitization such as posttranslational modification and protein translocation have also been suggested, but the molecular details are sketchy at this point (Solary *et al.*, 2001).

B. Molecular Mechanisms of Immunosensitization by Chemotherapeutic Drugs

1. UPREGULATION OF THE DEATH RECEPTORS

The findings by Friesen *et al.* (1996) that drugs induce apoptosis by regulation of death receptor signaling are not generalized. Studies by Yeh *et al.* (1998) show that in FADD deficient mice, the fibroblasts were resistant to FasL, TNF, and DR3 mediated apoptosis but were sensitive to DR4, oncogene, E1A, and c-myc and chemotherapeutic drugs. Likewise, targeted disruption of caspase-8 gene ablates apoptosis achieved by TNF receptors, FasL, and DR3 but not by UV, etoposide, ceramide, staurosporine, serum starvation, etc. (Varfolomeev *et al.*, 1998). These findings are corroborated by studies by Newton and Strasser (2000) who reported that lymphocytes from Fas-deficient *lpr* mice or transgenic mice expressing a dominant negative Fas associated death domain protein (FADD/MORT1) are as sensitive as normal lymphocytes to killing by gamma radiation and cytotoxic drugs such as cisplatin, doxorubicin, and etoposide.

The expression level of death receptors establishes the initial stage for the control of immune-mediated apoptosis. In the Fas system, various resistance mechanisms that negatively regulate the initiation of death signal from the Fas receptor (FasR) include overexpression of the receptor with mutations at the death domain region, loss of Fas expression, and alternative splicing
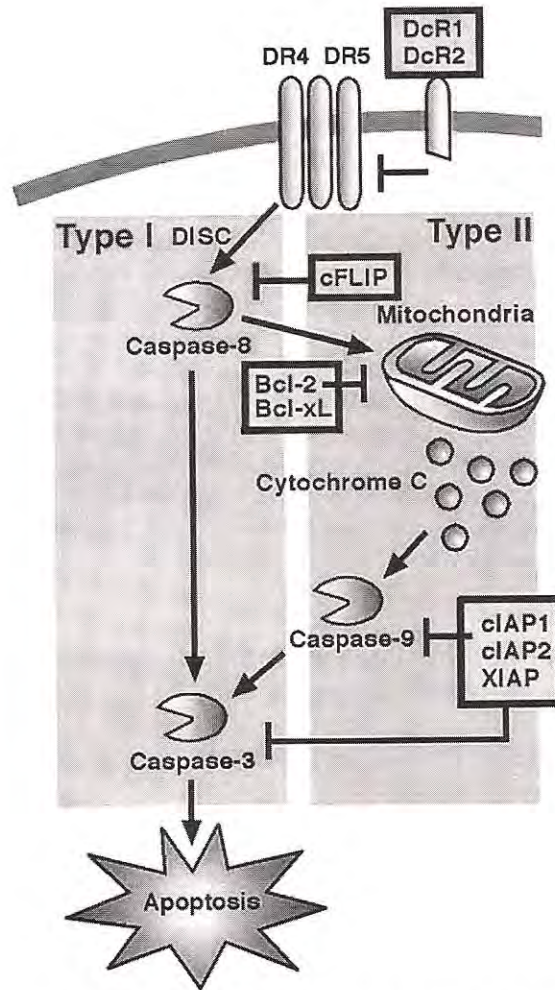
Fig. 2 Inhibition of death receptor-mediated apoptosis pathway by various antiapoptotic proteins. The death receptor-mediated apoptosis pathway is inhibited by several proteins (DcR1, DcR2, cFLIP, Bcl-2 family proteins, and IAP family proteins). Decoy receptors DcR1 and DcR2 can inhibit the signal by competitive binding with TRAIL. cFLIP inhibits activation of caspase-8 as a dominant negative form of caspase-8. Bcl-2 family proteins stabilize mitochondria and prevent the release of cytochrome c. IAP family proteins inhibit activation of caspase-9 and -3.

of the Fas receptor mRNA that generates the secreted soluble Fas (Eberstadt *et al.,* 1997; Martinez-Lorenzo *et al.,* 1998; Ruberti *et al.,* 1996). These defects lead to the reduction of functional receptors on the cell surface, thus decreasing the intensity of death signals. Similarly, in the Apo2L/TRAIL receptor system, the overexpression of decoy receptors (DcR1 and DcR2)

lacking the functional intracellular signaling domain or loss/mutation of the agonist receptors (DR4 and DR5) contributes to resistance to Apo2L/TRAIL-mediated apoptosis (Ashkenazi and Dixit, 1998).

To overcome the resistance to Fas and Apo2L/TRAIL-mediated apoptosis at the receptor level, treatment of certain tumors with subtoxic chemotherapeutic drugs upregulated the expression of the agonist receptors (FasR, DR5) while other alternatively spliced variants for soluble receptors or decoy receptors remained constant (Petak *et al.*, 2000; Uslu *et al.*, 1997). The upregulation of FasR and DR5 by drugs such as adriamycin, etoposide, CDDP, camptothecin, and 5-fluorouracil appears to be driven by an p53-dependent transcriptional activation (Nagane *et al.*, 2000; Wen *et al.*, 2000; Wang and Jeng, 2000). However, cells lacking functional wild-type p53 are equally susceptible to induction of agonist receptors by chemotherapeutic drugs, suggesting that an alternative p53-independent mechanism is also at work (Muller *et al.*, 1998). For example, the hyperexpression of ROS present in tumor cells has been shown to drive receptor expression (Friesen *et al.*, 1999). The mere upregulation of agonist receptors by drugs does not necessarily explain the molecular mechanisms of sensitization by drugs for death receptor-mediated apoptosis. Direct functional correlations need to be determined before concluding that upregulation of death receptor expression by drugs is responsible for sensitization to death receptor-mediated apoptosis.

2. UPREGULATION OF FADD AND Apaf-1 ADAPTOR PROTEINS

Following receptor trimerization, the recruitment of FADD protein to the intracellular death domains of the death receptors to form the DISC is required for the initiation of caspase activation. Inability to activate the initiator caspase such as caspase-8 through this process presents another possible scenario whereby resistance to death receptor-mediated apoptosis can arise (Peter *et al.*, 1997). The studies that examined mice lacking FADD and cells expressing dominant negative forms of FADD showed that the loss of and defect in FADD lead to inhibition of death receptor-mediated apoptosis (Zhang *et al.*, 1998; Yeh *et al.*, 1998; Wajant *et al.*, 1998). Furthermore, the loss of FADD expression accounts for the transformation of HBV-infected hepatocytes into hepatoma cells that are resistant to Fas-mediated apoptosis (Suzuki *et al.*, 1999).

Upregulation of FADD by drug-mediated sensitization has been observed in several tumor systems. In colon cancer and myeloblastoma, cisplatinum, doxorubicin and mitomycin C induce accumulation of FADD as well as Fas receptors (Micheau *et al.*, 1999a). Similar upregulation of FADD by cisplatinum and adriamycin was also observed in androgen-independent

prostate tumor cells (Ng *et al.*, 1998). Interestingly, chemotherapeutic drugs could also directly induce death receptor-mediated apoptosis by causing FADD receptor clustering independent of receptor–ligand crosslinking (Micheau *et al.*, 1999b). Taken together, chemotherapeutic drugs can potentially enhance the receptor death signaling by increasing the protein level of FADD and facilitating the formation of DISC.

While FADD serves as the necessary signaling bridge between the clustered receptors and initiator caspase-8, Apaf-1 is the essential link for mediating the mitochondrial death signaling that leads to activation of caspase-9. The mitochondrial apoptosis pathway stimulates the formation of apoptosome containing Apaf-1, cytochrome c, and pro-caspase-9, in response to various chemical stimuli or developmental cues (Zou *et al.*, 1999). Mice lacking Apaf-1 displayed insensitivity to apoptosis stimulated by chemotherapeutic drugs and developed severe birth defects such as brain and faciocranial deformities. Mutation of functional Apaf-1 is often found in malignant cancer cells, which render them resistant to chemotherapy (Yamamoto *et al.*, 2000). In addition, silencing of Apaf-1 gene expression by DNA methylation was observed in resistant malignant melanoma cells, and treatment with an inhibitor of DNA methylation restored the expression of Apaf-1 and sensitivity to apoptosis (Shinoura *et al.*, 2000).

Similar to FADD, Apaf-1 is also a target protein that is potentially upregulated by certain chemotherapeutic drugs. In adriamycin-sensitized multiple myeloma cells, a positive correlation was established between the upregulation of Apaf-1 by adriamycin and sensitivity of multiple myeloma cells to Apo2L/TRAIL (Jazirehi *et al.*, 2001). Furthermore, overexpression of Apaf-1 in resistant tumor cells by gene transfection enhances the apoptotic effects of chemotherapeutic drugs (Perkins *et al.*, 1998; Shinoura *et al.*, 2000). Clearly, expression of functional Apaf-1 is necessary for a successful antitumor therapeutic response.

3. DOWNREGULATION OF FLIP

One of the initial intracellular regulators for inhibiting the death receptor-mediated apoptosis is FLIP (FLICE-inhibitory proteins) (Thome *et al.*, 1997). FLIP is structurally similar to pro-caspase-8 but lacks a functional caspase catalytic site. FLIP functions as a dominant negative form of caspase-8 that inhibits the death receptor signaling by associating with pro-caspase-8 or the death receptor-FADD complex (DISC) and preventing the recruitment and activation of caspase-8 (also known as FLICE). The antiapoptotic role of FLIP was determined based on cells from FLIP-deficient mice that displayed hypersensitivity to death receptor-mediated apoptosis (Yeh *et al.*, 2000). Furthermore, in numerous cancer systems, resistance to death receptor-mediated apoptosis is positively correlated with the expression of

FLIP (Leverkus *et al.*, 2000; Elnemr *et al.*, 2001; Irisarri *et al.*, 2000; Panka *et al.*, 2001; Tepper and Seldin, 1999; Kim *et al.*, 2000). Resistance to death receptor-mediated apoptosis was also observed in cells that were transfected with the viral form of FLIP derived from herpes virus (Peter *et al.*, 1997; Bertin *et al.*, 1997; Hu *et al.*, 1997).

Chemotherapeutic drugs can also sensitize immunoresistant tumor cells by downregulation of FLIP. Drugs that inhibit protein or RNA synthesis, such as cyclohexamide and actinomycin D, can downregulate FLIP effectively and sensitize resistant tumor cells to Fas-mediated and Apo2L/TRAIL-mediated apoptosis (Leverkus *et al.*, 2000; Fulda *et al.*, 2000; Irisarri *et al.*, 2000; Griffith *et al.*, 1998). CDDP can also sensitize Fas-resistant osteosarcoma cells to Fas-mediated apoptosis, and this is correlated with the level of FLIP downregulation induced by CDDP (Kinoshita *et al.*, 2000). Downregulation of FLIP appears to be restricted to the "Type I" tumor cells that are capable of inducing a direct death receptor-mediated pathway (Fulda *et al.*, 2000). Hence, downregulation of FLIP might not be sufficient to sensitize "Type II" cells that primarily undergo the mitochondrial pathway induced by death receptors.

4. UPREGULATION OF PRO-CASPASES

Since caspase plays a central role in mediating apoptosis, it is reasonable to postulate that the level of pro-caspases dictates the threshold of tumor sensitivity to apoptosis. Indeed, the basal constitutive level of caspase gene transcription driven by the STAT-1 transcriptional factor maintains sensitivity to apoptosis mediated by TNF and IFN-γ (Chin *et al.*, 1997). Absence of STAT-1 led to low expression of caspases-1, -2, and -3 and resistance to TNFα-mediated apoptosis, while reconstitution of functional STAT-1 restored the expression of these caspases and sensitivity to apoptosis. The reduction of caspase-8 and caspase-10 mRNA expression by DNA methylation was observed in the apoptosis-resistant neuroblastoma cells. Similarly, restoration of caspase-8 gene expression by the methyltransferase inhibitor 5-aza-2'-deoxycytidine in the caspase-8 negative cells restored the sensitivity to Apo2L/TRAIL-mediated apoptosis (Eggert *et al.*, 2000; Grotzer *et al.*, 2000; Hopkins-Donaldson, 2000). This correlation between the lack of caspase-3 expression and the resistance to apoptosis was also observed in leukemia cells (Martinez-Lorenzo *et al.*, 1998).

While the methyltransferase inhibitor could serve as a sensitizing agent to Apo2L/TRAIL-mediated apoptosis by upregulating pro-caspase-8 and -10 expression, conventional chemotherapeutic drugs (etoposide, cisplatin, doxorubicin, and mitomycin C) could also sensitize tumor cells by selective induction of pro-caspase-8, -3 and -2 (Micheau *et al.*, 1999a; Droin *et al.*, 1998). The increase of pro-caspase-8, -3, and -2 appeared to be independent

of STAT-1 transcription (Micheau *et al.*, 1999a). The chemotherapeutic drug adriamycin also increased the expression of caspase-9 in the adriamycin-sensitized multiple myeloma cells to Apo2L/TRAIL-mediated apoptosis (Jazirehi *et al.*, 2001). The increase in the expression of initiator caspases such as caspase-8 and -9 is particularly important in reducing the apoptotic threshold since in most cells the levels of initiator caspases are low compared to those of the effector caspases such as caspase-3.

5. REGULATION OF Bcl-2 FAMILY PROTEINS

It is not clear whether the mitochondrial role is essential for the initiation for the caspase cascade or primarily acts as an amplifier system (Hengartner, 1998). Chemotherapeutic drugs and gamma radiation induce apoptosis in tumor cells. Overexpression of Bcl-2 or its prosurvival homologues or inactivation of Bax provide short-term protection against apoptosis. Caspases other than caspase-1 and -8 are essential inducers of drug-mediated apoptosis. Cells from caspase-8-deficient mice are normally sensitive to chemotherapeutic drugs and gamma radiation (Varfolomeev *et al.*, 1998) but those lacking caspase-9 are highly resistant (Hakem *et al.*, 1998; Kuida *et al.*, 1998). Strasser *et al.* (2000) proposed a Bcl-2 family function in which apoptotic stimuli cause disturbances in the mitochondria that leads to the release of cytochrome c and consequently caspase activation and that bcl-2 and homologues function by maintaining the integrity of the mitochondria.

The release of cytochrome c from the mitochondria into the cytoplasm is a crucial decisive event in the transduction of apoptosis (Green and Reed, 1998). In response to a myriad of environmental insults and internal damage, the mitochondria initiate cell suicide signals by releasing cytochrome c. Regulation of this important decision involves the Bcl-2 family that consists of proapoptotic members (Bax, Bcl-xS, Bak, Bad, Bok, Diva, Bik, Bid, Bim, Hrk, and Blk) and antiapoptotic members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, and Boo) (reviewed in Reed, 1999). The antiapoptotic members prevent the release of cytochrome c, while proapoptotic members induce the process. Currently, the exact molecular mechanism of the regulation of cytochrome c release by Bcl-2 family proteins is unclear. Three main models have been proposed to explain their possible mechanisms of action: (1) Bcl-2 members form mitochondrial membrane channels that facilitate protein transport, (2) Bcl-2 members associate with other proteins such as voltage-dependent anion channel (VDAC) to form protein transport channels, and (3) Bcl-2 members disrupt or maintain the integrity of the outer mitochondrial membrane and the membrane potential. The sensitivity of mitochondria to a variety of apoptotic stimuli is ultimately controlled by the relative ratio of the two types of Bcl-2 members (reviewed in Hengartner, 2000).

BH3-only proteins (members of the Bcl-2 family that have one of the Bcl-2 homology regions, BH3) are essential initiators of apoptosis. Many but not

all of the signals leading to caspase activation are regulated by the Bcl-2 protein family (Strasser *et al.*, 2000; Gross *et al.*, 1999). The mammalian Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1, and CED-9 all promote cell survival. A second subclass of proapoptotic Bcl-2 family members, the BH3-only proteins, mammalian Bad, Bic, Blk, Hrk/DR5, Bid, Bim, Noxa, and CEG-1 share amino acid sequence homology with Bcl-2 and each other only within the short 19–16 amino acids of the BH3 domains (Huang and Strasser, 2000). It has been proposed that certain death stimuli initiate apoptosis by disrupting mitochondrial integrity, releasing cytochrome c, and only then caspase activation. Accordingly, Bax-like proteins promote mitochondrial leakage and BH3-only proteins are thought to do likewise perhaps by indirectly interacting with Bax or Bak (Wei *et al.*, 2000). Thus, proximal Bcl-2 family members function instead to stabilize these membranes. Overexpression of Bcl-2 can promote cancer and autoimmunity and affects sensitivity of tumor cells to chemotherapeutic drugs. Mutation of BH3-only proteins can result in pathogenesis.

The role of Bcl-2 family proteins in the regulation of death receptor-mediated apoptosis has been controversial since its discovery. One school of thought believes that Bcl-2 antiapoptotic members (Bcl-2 or Bcl-xL) can block the Fas or Apo2L/TRAIL-mediated apoptosis in "Type II" cells, in which the mitochondrial apoptotic pathway (Type II pathway) is required for the death receptor-mediated apoptosis (Hinz *et al.*, 2000; Kawahara *et al.*, 1998; Kondo *et al.*, 1998; Mandal *et al.*, 1996). The opposing camp questions the existence of such "Type II" cells and believes that the mitochondrial pathway is dispensable for death receptor-mediated apoptosis and only serves to amplify the Fas-mediated death signaling (Memon *et al.*, 1995; Huang *et al.*, 1999; Keogh *et al.*, 2000; Kim *et al.*, 2001). Several lines of evidence have shown that chemotherapeutic drugs could induce the proapoptotic member Bax via a p53-dependent transcriptional mechanism (Zhang *et al.*, 2000; Oda *et al.*, 2000). In addition, the upregulation of Bax and downregulation of Bcl-xL induced by chemotherapeutic drugs are associated with immunosensitization to death receptor-mediated apoptosis (Mizutani *et al.*, 2001; Mori *et al.*, 1999). Because of the aforementioned controversy, it remains unclear if these changes in the Bcl-2 family proteins are responsible for immunosensitization.

6. DOWNREGULATION OF IAP FAMILY PROTEINS

IAP family proteins (Inhibitor of apoptosis) are a group of antiapoptotic proteins that function by directly inhibiting certain caspases. Expression of IAP proteins inhibits the "execution" phase of the death receptor-mediated apoptosis in resistant tumor cells since they have been shown to bind and inhibit the active form of caspase-3, a downstream caspase of the death receptor-mediated pathway (Yang and Li, 2000). IAP proteins also bind to

caspase-7 and -9, but not caspase-1, -6, -8, and -10, to inhibit apoptosis (Deveraux *et al.*, 1998). The binding of IAP proteins to caspases can be disrupted by Smac/DIABLO, which is an endogenous IAP neutralizing inhibitor released from mitochondria (Verhagen *et al.*, 2000; Du *et al.*, 2000). To date, eight members of IAP family proteins have been identified: NIAP, XIAP, cIAP1, cIAP2, Survivin, Livin, Apollon, and ML-IAP (Kuida *et al.*, 1998; Chen *et al.*, 1999; Ambrosini *et al.*, 1997; Vucic *et al.*, 2000; Kasof and Gomes, 2000; Liston *et al.*, 1996).

Overexpression of IAP proteins has been shown in a variety of chemoresistant human cancers. In particular, high protein expression of XIAP and cIAP-1 appears to be the most prevalent in many cancer cell lines (Tamm *et al.*, 2000). In AML patients, high levels of XIAP in the leukemia cells correlate with their poor survival rates (Tamm *et al.*, 2000). In melanoma, ML-IAP is also expressed in high levels in the tumor cells that are resistant to apoptosis induced by death receptors or drugs, in contrast to normal melanocytes (Vucic *et al.*, 2000). Clearly, overexpression of IAP family proteins negatively influences the antitumor therapeutic outcome by inhibiting apoptosis induced by drugs or death receptors. Thus, in order to improve the efficacy of antitumor immunotherapy, overcoming the resistance conferred by IAP family proteins may be necessary.

Subtoxic levels of chemotherapeutic drugs have been shown to reduce the activity of IAP family proteins in several tumor cell systems. In prostate cancer cells, actinomycin D preferentially downregulates XIAP and also sensitizes the cells to Apo2L/TRAIL-mediated apoptosis (Zisman *et al.*, 2001; Ng *et al.*, 1998). The reduction of XIAP by actinomycin D and cycloheximide was also linked to the sensitization to Fas-mediated apoptosis (Fulda *et al.*, 2000; Zisman *et al.*, 2001). In addition, the proapoptotic effects of the genotoxic drug 5-fluorouracil were correlated with the downregulation of cIAP-1 (Suzuki *et al.*, 1998). Overexpression of antisense XIAP or Smac/DIABLO (an endogenous protein inhibitor of IAP proteins) in the resistant ovarian and prostate cancer cells also sensitizes them to drug- or death receptor-mediated apoptosis, indicating that inactivation of IAP proteins is crucial for overcoming resistance (Aota *et al.*, 2000; Sasaki *et al.*, 2000).

## VII. CONCLUSIONS

A. A New Face for Chemotherapeutic Drugs

For more than 60 years after nitrogen mustard was introduced as the first antitumor chemotherapeutic drug, the main criterion for searching effective chemotherapeutic drugs has always been based on their direct cytotoxicity

against cancer. Indeed, the ideal drug would be the one that incurs the most damage. This idea has led us to believe that the higher the dosage of drugs is used against cancer, the more likely the cancer is cured. However, this general assumption of "the more, the better" may not be the most effective since such practice masks the subtle yet significant modulating effects on tumors when drugs are used at subtoxic concentrations. The studies in apoptosis and immunosensitization have revealed that subtoxic concentrations of chemotherapeutic drugs can elicit a variety of regulatory signals at many levels of the apoptotic process. These apoptotic regulatory effects may be distinct from their direct apoptosis-inducing functions. The aforementioned examples illustrate that certain chemotherapeutic drugs can sensitize resistant cancer cells to immune-mediated apoptosis by selectively downregulating antiapoptotic proteins or upregulating proapoptotic proteins involved in the pathway.

B. A Complementary Two-Signal Model as a New Antitumor Therapeutic Approach

Based on these recent findings, a two-signal model is proposed as a new antitumor approach for complementing the existing cancer immunotherapeutic strategies (Fig. 3). The model describes the synergistic induction of apoptosis caused by the complementary proapoptotic effects of chemotherapeutic drugs and immunocytotoxics. The immune component provides the essential apoptotic-triggering signal (signal II), whereas the drug component modulates the signaling proteins involved in the immune-mediated pathway (signal I). The death signal triggered by death receptors (signal II) proceeds down to a signaling block and it is not sufficient to complete the killing by itself. In order to allow the immune-induced death signal to proceed further downstream, a modifying signal provided by drugs (signal I) is required to fully activate immune-mediated apoptosis. There are two potential models for drug-mediated sensitizations. In model I, chemotherapeutic drugs provide the modifier signal (signal I) by selectively downregulating the specific block that hinders the immune-mediated pathway. Alternatively, the drugs can bypass the block by upregulating a proapoptotic factor. The upregulation of proapoptotic proteins lowers the signaling threshold for full activation of immune-mediated apoptosis.

The proposed model above demonstrates that tumor resistance to apoptosis can be reversed by two complementary proapoptotic signals such as chemotherapeutic drugs and TNF death-inducing ligands. Based on this model, we predict that other specific agents, such as peptide inhibitors or antisense that selectively regulate the apoptotic proteins involved in the immune-mediated apoptotic pathway, will also reverse the tumor resistance
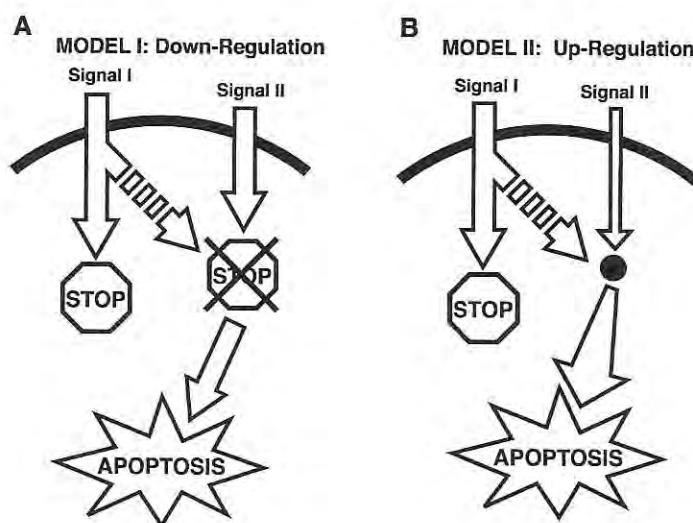
Fig. 3 Models of immunosensitization to death receptor-mediated apoptosis. A. Downregulation of antiapoptotic block by the modifying signal (Signal I). Both apoptotic pathways induced by Signal I and Signal II are blocked (indicated by the stop signs). Signal I exerts a distinct sensitizing effect, in addition to its apoptosis-inducing property. Signal I synergizes with Signal II by downregulating an apoptotic block in the Signal II (death-receptor)-mediated pathway, thereby completing a full activation of the death pathway. B. Upregulation of apoptotic signaling by the amplifying signal (Signal I). The threshold required to induce a full activation of Signal II is high. Signal I serves to amplify the death signal induced by Signal II by synergistically activating a signal target involved in the Signal II-mediated death pathway (indicated by black dot). Amplification of death signal allows full activation of the death pathway.

to immune-mediated apoptosis. Currently, tumor resistance to conventional therapies remains a major problem. The ideal choice for circumventing this problem should be an approach that is tumor selective, nontoxic, and effective in reversing tumor resistance. The immunotherapy-based two-signal complementation approach discussed here is potentially an effective approach that is nontoxic and tumor selective. The model will serve as the therapeutic framework for exploring other modifying agents that can enhance the therapeutic potential of preexisting or novel therapeutics such as Apo2L/TRAIL against immune-resistant or drug-resistant cancers.

ACKNOWLEDGMENT

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Synergy Is Achieved by Complementation With Apo2L/TRAIL and Actinomycin D in Apo2L/TRAIL-Mediated Apoptosis of Prostate Cancer Cells: Role of XIAP in Resistance

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BACKGROUND. Tumors have an inherent immunogenicity that can be exploited by immunotherapy. However, often tumors develop mechanisms that render them resistant to most immunologic cytotoxic effector mechanisms. This study examines the underlying mechanism of resistance to Apo2L/TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)-mediated apoptosis.

METHODS. We studied prostate tumor cell lines for their sensitivity to Apo2L/TRAIL-mediated apoptosis in the presence and absence of the sensitizing agent actinomycin D (Act D). Apoptosis was determined by flow cytometry and signaling for apoptosis by Western blot.

RESULTS. Treatment with subtoxic concentrations of Act D significantly sensitizes the tumor cells (CL-1, DU-145, and PC-3 prostate tumor cells) to Apo2L/TRAIL-mediated apoptosis. The cytotoxicity of Act D-sensitized prostate tumor cells was a result of synergistic activation of caspases (caspase-3, -9, and -8), detectable after 6 hr of treatment. Treatment with Apo2L/TRAIL alone, although it was insufficient to induce apoptosis, resulted in the loss of mitochondrial membrane potential and release of cytochrome c from the mitochondria into the cytoplasm in the absence of significant caspases activation. These findings suggested that a major apoptosis resistance factor blocking the Apo2L/TRAIL apoptotic signaling events is present downstream of the mitochondrial activation. The expression of receptors and anti-apoptotic proteins were examined in Act D-sensitized CL-1 cells. The earliest and the most pronounced change induced by Act D was down-regulation of X-linked inhibitor of apoptosis (XIAP) and up-regulation of Bcl-xL/-xS proteins. The role of XIAP in resistance was demonstrated by overexpression of Smac/DIABLO, which inhibited inhibitors of apoptosis (IAPs) and sensitized the cells to Apo2L/TRAIL. Apo2L/TRAIL receptors (DR4, DR5, DcR1, and DcR2), c-FLIP, Bcl-2, and other IAP members (c-IAP1 and c-IAP2) were marginally affected at later times in the cells sensitized by Act D.

CONCLUSION. This study suggests that the combination of Act D-induced down-regulation of XIAP (Signal I) and Apo2L/TRAIL-induced release of cytochrome c (Signal II) leads to the reversal of resistance to Apo2L/TRAIL-mediated apoptosis in the tumor cells. The sensitization

INTRODUCTION

The tumor necrosis factor (TNF) ligand superfamily serves an important role in the host immune defense against cancer as an inducer of apoptosis in tumor targets [1]. The death-inducing TNF ligand members (TNFα, FasL/Apo1L/CD95L, TNF-related apoptosis-inducing ligand [Apo-2L/TRAIL], and Apo-3L/DR3L/Tweak), present on the immune cell surface as type II membrane proteins or secreted in soluble form, can mediate apoptosis in sensitive target cells by binding to their cognate death receptors (TNFRI, Fas/Apo-1/CD95, DR4/TRAIL-R1, or DR5/TRAIL-R2, DR3/TRAMP/WSL-1) on the target cell surface [2–6]. Among the TNF ligand members, Apo2L/TRAIL recently has drawn the most research interest as a potential effective anti-tumor therapeutic agent. Apo2L/TRAIL has been shown to induce rapid apoptosis in wide variety of cancer cell lines [6,7]. In addition, Apo2L/TRAIL is postulated to be selectively cytotoxic against transformed cells and not against the majority of normal cells [7–10]. The Apo2L/TRAIL decoy receptors (DcR1/TRID/TRAIL-R3, DcR2/TRUNDD/TRAIL-R4) lack the functional cytoplasmic death domain that is needed for positive apoptotic signaling and have been postulated to protect normal cells from Apo2L/TRAIL-induced apoptosis by competitive binding with the agonist receptors (DR4/TRAIL-R1, DR5/TRAIL-R2) [10]. Although TNF-α and FasL/Apo1L/CD95L elicit toxicity in normal tissues, in vivo studies in mice and primates also show that Apo2L/TRAIL is tolerated well [7,8].

The molecular mechanism of Apo2L/TRAIL-mediated apoptosis is thought to be similar to the death receptor-mediated apoptosis pathway of TNFα and FasL/Apo1L/CD95L [10,11]. Like TNFα, Apo2L/TRAIL initiates the apoptotic signal upon binding to its cognate death receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2) and induces clustering of the receptors and formation of a death-inducing signaling complex (DISC). The complex recruits multiple pro–caspase-8 molecules by means of death domain interactions with the adaptor molecule FADD [12,13]. The close proximity of pro–caspase-8 induced by the complex leads to autoactivation of pro–caspase-8, which in turn leads to the caspase activation cascade involving the activation of effector caspase-3. This rapid pathway involving the direct activation of caspase-3 from caspase-8 is termed the type I pathway [14]. Alternatively, the death pathway can be further amplified or redirected by means of activation of the mitochondrial pathway (the type II pathway) [14,15]. Recent evidence has shown that Apo2L/TRAIL also induces events of the mitochondrial pathway such as release of cytochrome c, depolarization of mitochondrial membrane potential, and activation of caspase-9 [15–17]. Apo2L/TRAIL-activated caspase-8 can generate truncated Bid, which in turn triggers the mitochondria to release cytochrome c, leading to the assembly of the apoptosome (an octamer complex of cytochrome c, Apaf-1, pro–capase-9, and other unidentified proteins). Formation of the apoptosome activates pro–caspase-9, which then feeds back to the core effector caspase cascade involving caspase-3 [18]. In both pathways, successful activation of the effector caspase cascade is required to result in the final apoptotic phenotype. It is generally believed that inhibition of the central apoptotic program involving caspase activation leads to tumor resistance to apoptosis.

Tumor resistance to conventional therapies such as chemotherapy or radiation presents a major problem in cancer treatment today. Failure to eradicate advanced resistant tumors with conventional therapies has led to exploration of novel therapeutic approaches such as immunotherapy or gene therapy. Immunotherapy is generally considered for the generation of anti-tumor cytotoxic lymphocytes that can recognize and eradicate the drug-resistant tumor cells. Also, immunotherapy is predicated on the notion that all drug-resistant tumors will succumb to cytotoxic lymphocytes. Cytotoxic lymphocytes kill by various mechanisms, including perforin/granzymes and the TNF-α superfamily that kill by apoptosis [19]. Also, chemotherapeutic drugs kill by apoptosis. Because immunotherapy may use apoptosis to eradicate tumors similar to chemotherapy and radiation, chemoresistant tumor cells are most likely resistant to immunotherapy or any other novel apoptosis-inducing therapies [20]. Indeed, TNF-related ligands, which share certain components of an apoptotic pathway induced by chemotherapeutic drugs, can also become ineffective to kill chemoresistant tumor cells [21]. To circumvent the problem of tumor immune resistance, one must explore alternatives to overcome the inhibition of the central apoptotic program to complement novel immunotherapeutics against resistant tumors.

The problem of tumor resistance to various immune cytotoxic cells/factors has been reported in many systems. Recently, we and others have reported that subtoxic levels of chemotherapeutic drugs sensitize chemoresistant and immunoresistant tumor cells to Apo2L/TRAIL-mediated cytotoxicity [22–28]. In particular, we have found that actinomycin D (Act D) is a potent sensitizer to Apo2L/TRAIL-mediated apoptosis in AIDS-related Kaposi sarcoma cells [23]. We hypothesize that Act D can complement Apo2L/TRAIL-mediated apoptosis by removing labile apoptotic inhibitors in the Apo2L/TRAIL signaling pathway. This study examined the immune resistance of tumor cells to Apo2L/TRAIL-mediated apoptosis and sensitization by chemotherapeutic drugs. The following were investigated: (1) the differences in the apoptotic signaling induced by Apo2L/TRAIL, Act D, and combination; (2) the characterization of the anti-apoptotic resistance gene product; and (3) the determination of the role of the anti-apoptotic gene product in resistance to TRAIL-mediated apoptosis.

MATERIALS AND METHODS

Cells and Reagents

The human prostate tumor cell lines DU-145 and PC-3 were purchased from ATCC (Manassas, VA). The CL-1 cell line was kindly provided by Dr. Arie Belldegrun. The establishment and characterization of CL-1 cells were described in Tso et al. [29]. The prostate cells were cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Woodland, CA). Actinomycin D (Act D) was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human Apo2L/TRAIL was kindly provided by Dr. Avi Ashkenazi (Genentech, South San Francisco, CA).

DNA Fragmentation Assay

The following propidium iodide-based flow cytometric DNA fragmentation assay was adopted from Nicoletti et al. [30]. A total of 2×10^5 prostate tumor cells were seeded in each well in 12-well plates in RPMI 1640 medium supplemented with 10% FBS overnight. Recombinant Apo2L/TRAIL (10 ng/ml) and Act D (100 ng/ml) were simultaneously added to the cell cultures, and the samples were collected at various time periods after the addition of Act D and Apo2L/TRAIL. For sample collection, both the adherent cells and floating dead cells in the culture supernatant were collected. The adherent cells were detached with 1 ml of phosphate buffered saline (PBS) supplemented with 0.5 mM ethylenediaminetetraacetic acid (EDTA) in each well. The samples were then fixed and permeabilized in 2 ml of cold 70% ethanol for at least 1 hr at -20°C. After fixation with ethanol, the samples were washed twice with 2 ml of PBS to remove the ethanol and stained with 0.1 ml per sample of DNA staining solution (PBS supplemented with 50 µg/ml propidium iodide and 50 µg/ml RNAse A) at 37°C for 30 min. The final volume is brought up to 1 ml by adding 0.9 ml of PBS. Measurement of DNA fragmentation in propidium iodide-stained cells was performed by using an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). Region markers were drawn for Sub-G1, G0/G1, S, and G2/M populations for quantitation of the cell populations by the flow cytometer. The Sub-G1 population represents the cells containing DNA hypoploidy, a characteristic of apoptotic cells undergoing DNA fragmentation.

Measurement of Mitochondrial Membrane Depolarization

The mitochondria-specific dye 3,3′-dihexyloxacarbocyanine (DiOC$_6$(3)); Molecular Probes, Inc., Eugene, OR) was used to measure mitochondrial energization. Prostate tumor cells were grown in six-well plates (1×10^6 in 1 ml of RPMI 1640 medium supplemented with 10% charcoal/dextran-treated FBS) and were treated with Apo2L/TRAIL (10 ng/ml) and/or Act D (100 ng/ml) simultaneously. After treatments, the cells were collected at various time periods. A total of 50 µl of 40 µM DiOC$_6$(3) was loaded to stain the cells for 30 min immediately after the cells were collected. The cells were detached by using PBS supplemented with 0.5 mM EDTA, then washed twice in PBS, resuspended in 1 ml of PBS, and subjected to flow cytometric analysis (FL1) by using an Epics XL flow cytometer (Coulter Electronics, Inc.).

Isolation of Cytosolic and Mitochondrial Fractions and Determination of Cytochrome c Release and Smac/DIABLO

Prostate tumor cells were grown to near confluence (approximately 8.0×10^6 cells) in 100×20 mm culture dishes. After treatment with Act D (100 ng/ml) and/or Apo2L/TRAIL (10 ng/ml), both the floating and attached cells were collected. The cell pellets were washed twice in cold PBS then were resuspended in 2 volumes of homogenization buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM sodium EDTA, 1 mM sodium ethyleneglycoltetraacetic acid, 1 mM dithiothreitol, and a tablet of Mini complete protease inhibitor cocktail (Roche, Indianapolis, IN) per 10 ml of homogenization buffer). Cells were incubated on ice for 30 min to let the cells swell and lyse. Cell homogenates were prepared by disrupting the cells by 40 strokes in a 2-ml Dounce glass homogenizer with

a loose-size pestle. Unbroken cells and nuclei were centrifuged down at $2,500 \times g$ at 4°C for 5 min twice. Supernatants, which contained mitochondria, were collected and subjected to further centrifugation at $16,000 \times g$ at 4°C for 30 min to pellet down mitochondrial fraction. The postmitochondrial supernatants were successively filtered through 0.2 μm and 0.1 μm Ultrafree MC filters (Millipore, Bedford, MA) to yield a cytosolic fraction. The cytosolic fraction was then subjected to Western blotting analysis for cytochrome c and Smac/DIABLO.

Antibodies and Western Blot Analysis

Tumor cells were treated with Act D (100 ng/ml) then collected at various time periods. The cells were lysed at 4°C in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl), supplemented with a tablet of Mini protease inhibitor cocktail (Roche). The cell lysates (40 μg) were separated on 10% or 15% sodium dodecyl sulfate–polyacrylamide gel (Bio-Rad, Hercules, CA). The proteins were blotted onto Hybond nitrocellulose membranes (Amersham, Arlington Heights, IL) and blocked with 10% w/v nonfat dry milk in Tris Buffered saline (TBS)/Tween (0.05% Tween-20 in TBS). After washing once with TBS/Tween, the blots were incubated with primary antibodies in 10 ml of the 5% blocking solution (5% w/v nonfat dry milk in TBS/Tween) for 1 hr in room temperature. After the primary antibodies are removed, the blots were washed three times with TBS/Tween, then incubated with horseradish peroxidase-coupled mouse or rabbit secondary antibodies (1:10,000) in the 5% blocking solution for 1 hr at room temperature, washed three times again, and developed with a chemiluminescence reagent, LumiGlo (New England Biolab, Beverly, MA). The polyclonal anti-Apo2L/TRAIL receptors (DR4, DR5, DcR1, DcR2) antibodies were purchased from Calbiochem (San Diego, CA). The monoclonal anti-actin antibody was purchased from Chemicon (Temecula, CA).

The polyclonal antibodies against inhibitors of apoptosis (IAPs) family proteins (c-IAP1, c-IAP2, and X-linked inhibitor of apoptosis [XIAP]) were purchased from Trevigen (Gaithersburg, MD). The anti–Bcl-2 monoclonal antibodies, anti–Bcl-x polyclonal antibodies, and anti–c-FLIP antibodies were purchased from Santa Cruz (Santa Cruz, CA), BD Pharmingen (San Diego, CA), and Upstate Biotechnology (Lake Placid, NY), respectively. The densitometric analyses of the immunoblots was performed on an iMac computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Transient Transfection of Tumor Cells and the pcDNA3-Flag-Smac Construct and Immunoblotting of Caspases and Smac

The expression construct pcDNA-flag-smac was kindly provided by Dr. Xiaodong Wang. The control vector pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA). The transfection of prostate tumor cells was performed by using the poly-cationic liposome reagent lipofectAMINE 2000 (Life Technologies, Rockville, MD). The transfection was done according to the manufacturer's procedures. The vector DNA and pcDNA3-Smac/DIABLO were first mixed with the liposome reagent in the ratio of 2.0 μl of lipofectAMINE 2000 to 1.25 μg of DNA in 0.5 ml of serum-free RMPI 1640 medium (Mediatech) for 20 min in room temperature. The 0.5-ml liposome DNA mixture was then added to the cells plated on 24-well plates for transfection. After 6 hr, the transfection medium was removed and fresh medium containing 10% FBS was added to allow the recovery of the cells. The monoclonal anti-Flag antibody M2 clone was purchased from Sigma for the detection of flag-Smac in immunoblotting assays. The antibodies against active caspase-3, poly-ADP-ribose-polymerase (PARP) were purchased from Pharmingen and were used for detection of caspase activation.

RESULTS

Treatment With a Combination of Soluble Human Apo2L/TRAIL and Act D Synergistically Induces Apoptosis in the Androgen-Independent Prostate Cancer Cell Lines

We assessed the cytotoxic effect of a combination of recombinant soluble human Apo2L/TRAIL and Act D against the androgen-independent prostate tumor cell lines (CL-1, DU-145, and PC-3). In a recent report, we have titrated the agents to determine optimal levels of cytotoxicity [31]. In a 24-hr DNA fragmentation assay, we first measured the cytotoxicity of CL-1 cells mediated by Apo2L/TRAIL alone (10 ng/ml), Act D alone (100 ng/ml), or a combination of Apo2L/TRAIL (10 ng/ml) and Act D (100 ng/ml). As shown in Figure 1A, the CL-1 cells were resistant to Apo2L/TRAIL alone or Act D alone in 24 hr, because these treatments resulted in little DNA fragmentation compared with the untreated cells. The CL-1 cells remain resistant (less than 7% of Sub-G1 cells) after 48- and 72-hr treatments with either agent alone (data not shown). However, a combined treatment with Apo2L/TRAIL and Act D for 24 hr induced DNA fragmentation in 65% of the cell population (Fig. 1A).

Next, we characterized the kinetics of cell death in CL-1, DU-145, and PC-3 cells induced by Act D alone, Apo2L/TRAIL alone, and a combination of Apo2L/
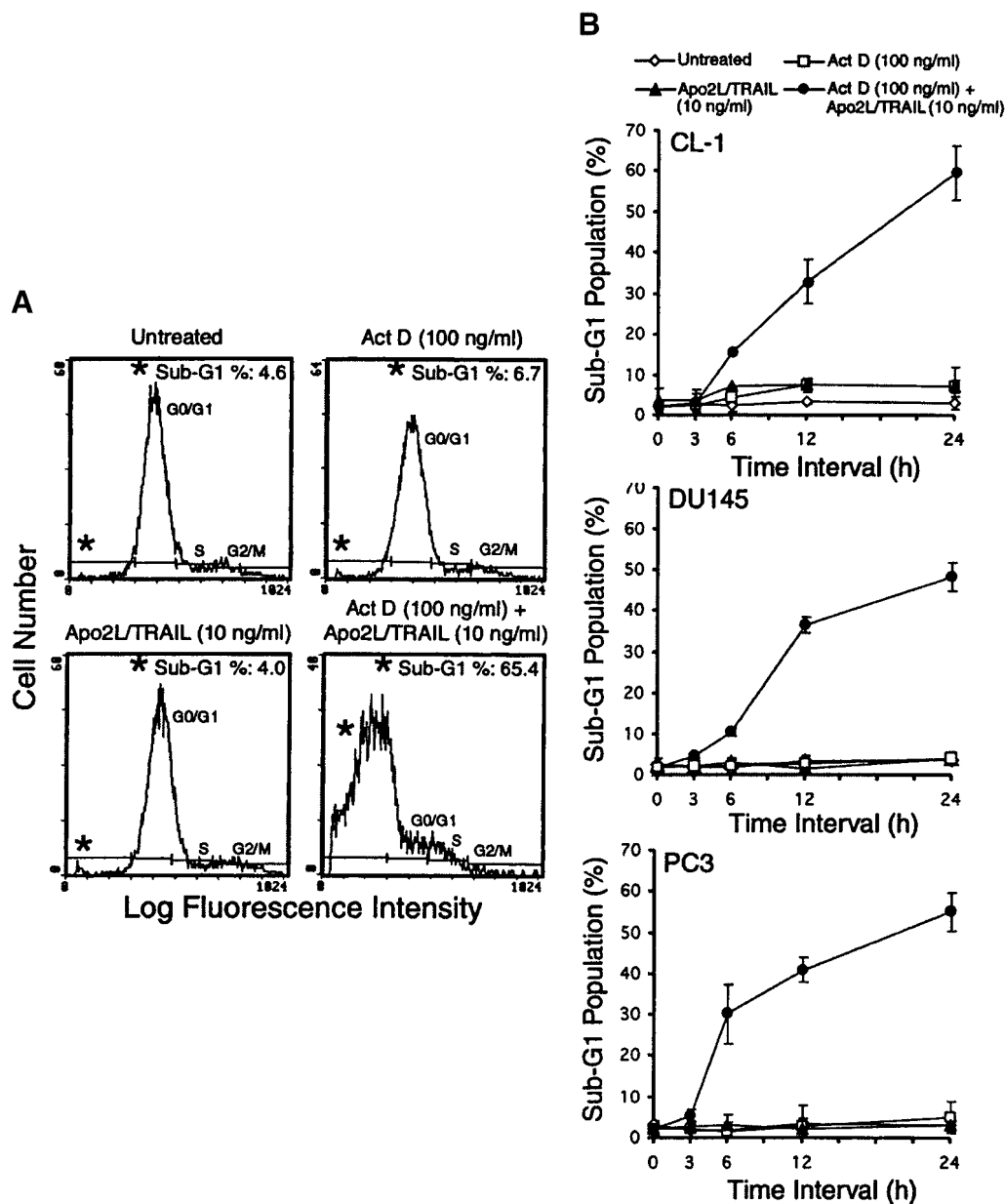
Fig. I. Synergistic induction of apoptosis in prostate tumor CL-I cells by treatment with combination of tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL) and actinomycin D (Act D). The cells were treated with Act D (I00 ng/ml), Apo2L/TRAIL (I0 ng/ml), combination of both, or left untreated. The percentages of apoptotic cells were determined by a flow cytometric cell cycle analysis based on propidium iodide staining. The percentage of cells in each cell cycle phase (Sub-GI, G0/GI, S, and G2/M) was gated and determined by the flow cytometer. The Sub-GI population (indicated by asterisks) represents the apoptotic cells containing hypoploid DNA content. **A**: Apoptotic effects of Act D (I00 ng/ml), Apo2L/TRAIL (I0 ng/ml), and combined treatment with both on CL-I cells after 24 hr, measured in the amount of cells undergoing DNA fragmentation (Sub-GI population). **B**: Apoptotic effects of Act D (I00 ng/ml), Apo2L/TRAIL (I0 ng/ml), and combined treatment with Act D (I00 ng/ml) and Apo2L/TRAIL (I0 ng/ml) on CL-I, DU-I45, and PC-3 cells at various time intervals. The data points represent the percentages of Sub-GI populations at various time points in mean ± SD (n = 3). The cells were harvested and fixed at different time intervals, then all samples were accumulated, fixed, and quantitated in a flow cytometer concurrently.

TRAIL and Act D. When CL-1 cells were treated with 100 ng/ml Act D alone or 10 ng/ml Apo2L/TRAIL alone, the spontaneous cytotoxicity (6%) remained constant throughout the 24-hr time course. When CL-1 cells were treated with a combination of Apo2L/TRAIL (10 ng/ml) and Act D (100 ng/ml) simultaneously, the

earliest increase in DNA-fragmented cell population was noticed at the 6th hr. By the 12th hr, the percentage of DNA-fragmented cells rose to 35% and reached the maximum at 65% by the 24th hr (Fig. 1B). These results suggest that the sensitizing effect of Act D has taken place by the 6th hr and the onset of apoptosis has also

begun at this time. In DU-145 and PC-3 cells, we observed a similar synergistic induction of apoptosis by the combination treatment (Fig. 1B). Both cell lines reached a lower percentage of killing at 24 hr than that of CL-1 cells (48.4 and 55.2%, respectively, Fig. 1B). Like CL-1 cells, notable increase of cell death was observed starting at the 6th hr in DU-145 and PC-3 cells. Normal cells were not sensitized by Act D for TRAIL-mediated apoptosis [32]. Because CL-1 cells achieved the highest synergistic apoptotic killing among the three cell lines examined, we chose to study this cell line for the following signaling studies.

Treatment With a Combination of Act D and Apo2L/TRAIL Leads to Activation of Caspases- 8, -9, and -3

Activation of caspases is an essential signaling step in Apo2L/TRAIL-mediated apoptosis, because caspase inhibitors have been shown to block Apo2L/TRAIL-induced apoptosis [6]. Apo2L/TRAIL triggers a sequential activation of caspase-8 then caspase-3, similar to the type I Fas-mediated apoptotic pathway [11,14]. Apo2L/TRAIL can also trigger a type II pathway that involves the participation of the mitochondria in amplifying the death signal and the activation of caspase-9 [15,17]. We also examined whether caspase-8, -9, and -3 were activated when CL-1 cells were treated with Apo2L/TRAIL alone, Act D alone, or a combination of Apo2L/TRAIL and Act D. We examined the presence of cleaved PARP, which is a substrate of active caspase-3 when a cell undergoes apoptosis. As shown in Figure 2, PARP was cleaved in the cells

that were treated with a combination of Apo2L/TRAIL and Act D, and the appearance of cleaved PARP (p85) was observed at the 6th hr, which correlates with the time when the cells began to undergo DNA fragmentation. Caspase-8, -9, and -3 were also activated in samples that were treated with a combination of Apo2L/TRAIL and Act D, whereas Apo2L/TRAIL alone and Act D alone did not lead to activation of caspases even at 24 hr. The inability to activate caspases when the CL-1 cells were treated with Apo2L/TRAIL alone or Act D alone correlates to the unresponsiveness of CL-1 cells to apoptosis induced by either agent alone. Furthermore, the activation of caspase-8, -9, and -3 also began at the 6th hr, which corresponds to the time when we initially observed the cleavage of PARP. For all samples, the level of actin was also examined to ensure equal loading of the samples in the blot. Together, these results demonstrate that Apo2L/TRAIL-induced apoptosis in Act D-sensitized CL-1 cells undergoes a caspase-dependent apoptotic pathway.

Both the Apo2L/TRAIL Alone and Combination Treatments Induce the Depolarization of Mitochondrial Membrane Potential and Release of Cytochrome c From the Mitochondria

Mitochondria play a pivotal role in the intracellular signaling of apoptosis [32]. The disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) and the release of cytochrome c from mitochondria into the cytoplasm are associated with apoptosis induced by chemotherapeutic drugs and TNF-related ligands [16,32,33]. In the Apo2L/TRAIL-mediated apoptosis, after the activation
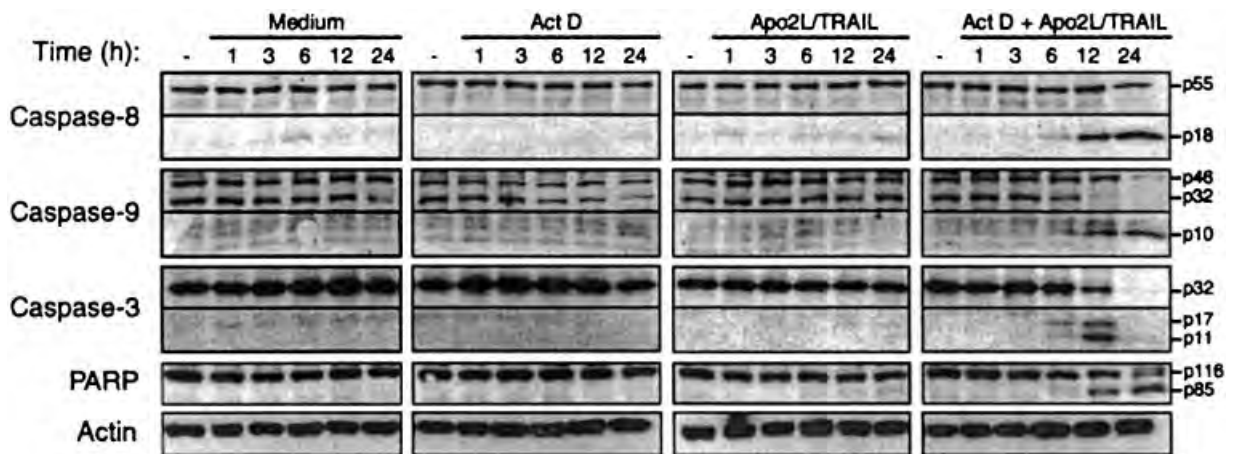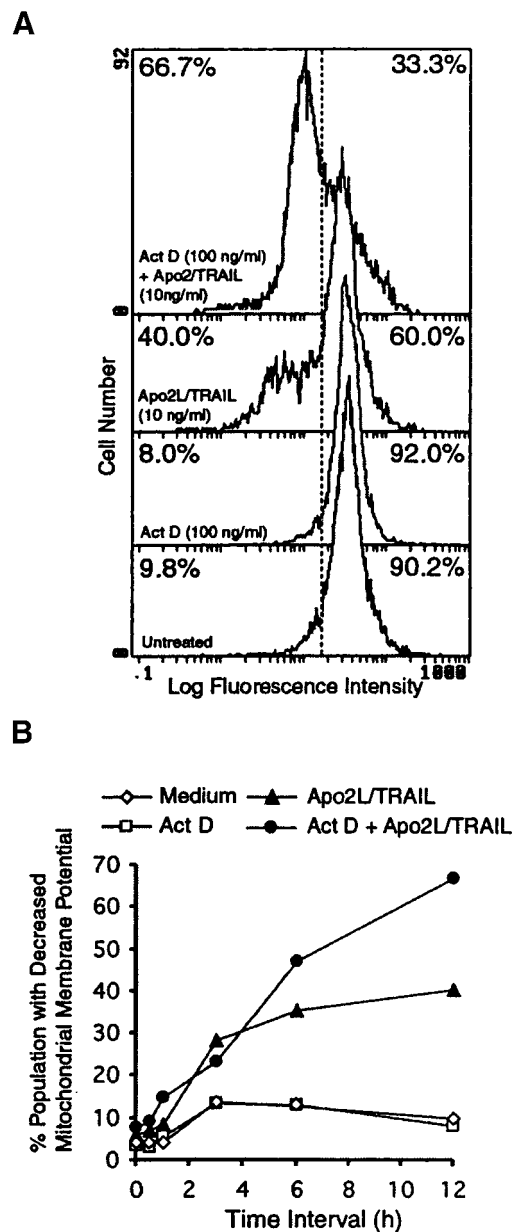

Fig. 2. Tumor necrosis factor−related apoptosis-inducing ligand (Apo2L/TRAIL) -induced caspase activation in actinomycin D (Act D)-treated and untreated CL-1 cells. The cells treated with Act D (100 ng/ml), Apo2L/TRAIL (10 ng/ml), both Act D and Apo2L/TRAIL, and control medium were harvested at different incubation times for immunoblotting analysis. A panel of polyclonal antibodies detected the following caspase proteins and poly-ADP-ribose-polymerase (PARP): caspase- 8 proform (p55) and its cleaved activated subunit (p18); zymogen pro−caspase-9 (p46), a variant of pro−caspase-9 (p32), and cleaved activated subunit (p10); zymogen pro−caspase-3 (p32), and cleaved activated subunits (p17 and p11); and intact PARP protein (p116) and PARP cleavage product (p85). Beta-actin was also detected to ensure equal loading of the samples.

of initiator caspases, mitochondria release cytochrome c into the cytoplasm to form an apoptosome (an octamer complex of Apaf-1, cytochrome c, ATP, and pro–caspase-9) [18]. Formation of this complex activates caspase-9 and subsequently activates downstream effector caspases [32]. In the following experiments, we examined the involvement of the loss of the mitochondrial membrane potential and the release of cytochrome c in the apoptosis induced by Act D plus Apo2L/TRAIL. We observed after 12 hr that CL-1 cells that were treated with Apo2L/TRAIL plus Act D resulted in the highest percentage of mitochondrial depolarization at 67% (Fig. 3A). Surprisingly, Apo2L/TRAIL alone, although it did not activate caspases in CL-1 cells, induced 40% of mitochondrial depolarization (Fig. 3A). Act D alone did not induce significant level of mitochondrial depolarization after 12 hr. The mitochondrial depolarization seen in both the Apo2L/TRAIL-alone–treated cells and the combination-treated cells appears to be an early event as it began to occur after 3 hr (Fig. 3B). Apo2L/TRAIL alone induced significant mitochondrial depolarization very early on, despite lack of caspase activation and DNA fragmentation (Fig. 3B).

The instability of mitochondria induced by Apo2L/TRAIL alone and Apo2L/TRAIL plus Act D was also confirmed by the release of cytochrome c. In the next experiment, we extracted the cytosolic fraction from the same batch of cells treated for the DNA fragmentation experiment seen in Figure 1B and analyzed the level of the released cytochrome c in the cytosolic fraction. We observed that both the Apo2L/TRAIL-treated and combination-treated cells released cytochrome c into their cytoplasm (Fig. 4). Act D alone did not induce the release of cytochrome c. The release of cytochrome c could be seen earliest at the 6-hr treatment of Apo2L/TRAIL alone (10 ng/ml) and at the 3-hr treatment of Apo2L/TRAIL (10 ng/ml) plus Act D (100 ng/ml) combination. Even though a significant level of the cytochrome c release was observed at 12 hr for both TRAIL-treated cells, only the Act D plus Apo2L/TRAIL treatment resulted in significant DNA fragmentation. These results demonstrate that Apo2L/TRAIL signaling is activated through the cytochrome c release stage and further, these findings indicate that the tumor resistance to Apo2L/TRAIL could occur downstream of cytochrome c-release.

Changes in the Protein Level of Apo2L/TRAIL Receptors and Various Anti-Apoptotic Proteins Induced by Act D

Recent reports have documented that chemotherapeutic drugs up-regulate or induce the expression of the agonist Apo2L/TRAIL receptors (DR4, DR5) to

Fig. 3. Tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL)-induced depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in actinomycin D (Act D)-treated and untreated CL-1 cells. **A**: Depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in both Apo2L/TRAIL-treated samples after 12 hr of treatments. The cells were treated in four conditions (untreated, 100 ng/ml Act D, 10 ng/ml Apo2L/TRAIL, and combination of 100 ng/ml Act D plus 10 ng/ml Apo2L/TRAIL) and harvested after 12 hr of treatment. The cells were stained in $DIOC_6(3)$ for 30 min then subjected to reading in a Coulter Epics-XL flow cytometer. The depolarized events with lower ($\Delta\Psi_m$) were gated on the left of the dash line and normal events are located to the right. The cellular events were quantitated on FL1 fluorescence channel. **B**: Kinetics of $\Delta\Psi_m$ depolarization over a 12-hr course. Cells were treated by the various indicated conditions and harvested at different time points over a 12-hr time course. Samples were measured after 30 min of $DIOC_6(3)$ incubation after the indicated incubation time period.
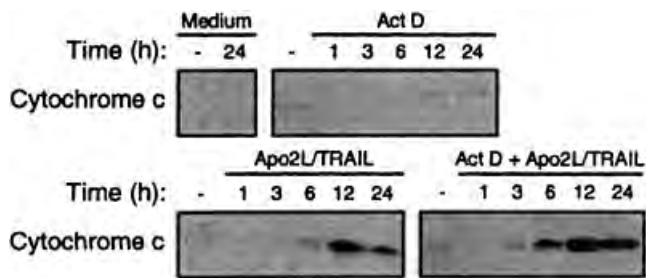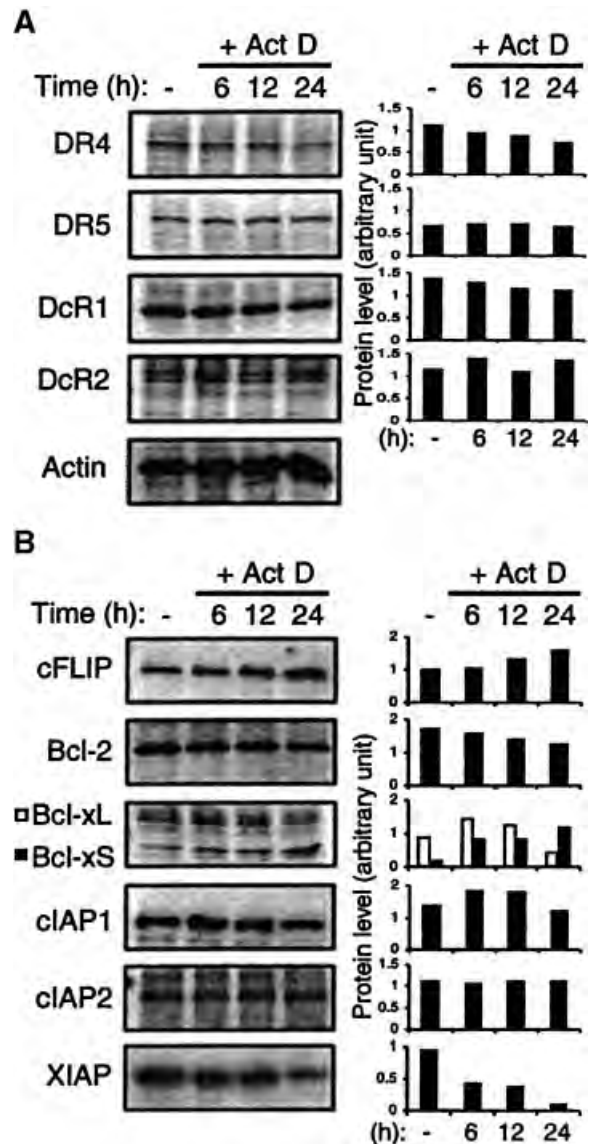
Fig. 4. Tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL)-induced mitochondrial events in actinomycin D (Act D)-treated and untreated CL-1 cells. A. Treatments with Apo2L/TRAIL (10 ng/ml) or both Act D (100 ng/ml) and Apo2L/TRAIL (10 ng/ml) increased the level of cytosolic cytochrome c-release in a time-dependent manner. Cytosolic fractions were extracted from the treated cells at various time intervals and subjected to immunoblotting analysis. Cytochrome c was detected as a 15-kDa band.

Fig. 5. Effects of actinomycin D (Act D) treatment on the expression of tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL) receptors and various anti-apoptotic proteins. **A**: Effects of Act D on the expression of Apo2L/TRAIL receptors DR4 and DR5, and decoy receptors DcR1 and DcR2. Protein lysates were prepared from cells treated with Act D (100 ng/ml) at 6-, 12-, and 24-hr time points, and receptor expressions were determined by immunoblotting analysis. β-Actin was determined to ensure equal loading of protein. The level of protein expression was measured by using the NIH Image software and plotted in arbitrary units. **B**: Effects of Act D on the expression of various anti-apoptotic proteins (c-FLIP, Bcl-2, Bcl-x, c-IAP1, c-IAP2, and XIAP).

result in a synergistic Apo2L/TRAIL-mediated cytotoxicity [25,26,34–36]. Because Act D sensitized prostate tumor cells to Apo2L/TRAIL-mediated apoptosis, we examined whether Act D affects the protein expression of both the agonistic receptors DR4 and DR5 and the antagonistic decoy receptors DcR1 and DcR2. CL-1 cells were treated at 6, 12, and 24 hr with Act D alone, and cell lysates were extracted and analyzed by using immunoblotting. As seen in Figure 5A, Act D down-regulated DR4, DcR1 receptor expression, but affected DR5 expression minimally.

We also analyzed the expression of DR4 and DR5 by flow cytometry. The surface expression of DR4 was slightly down-regulated after treatment with 100 ng/ml of actinomycin D. However, the expression of DR5 was slightly up-regulated by actinomycin D (the mean fluorescence of untreated CL-1 cells was 19.3 and for the actinomycin D-treated cells was 32.0; data not shown). The level of DcR2 fluctuated in a 24-hr course but still remained relatively high at 24 hr.

Next, we examined the protein expression of Bcl-2 family members (Bcl-2, Bcl-xL, and Bcl-xS) and caspase inhibitory molecules (c-FLIP, c-IAP1, c-IAP2, and XIAP). Bcl-2 and Bcl-xL negatively regulate the sensitivity to apoptosis by inhibiting the mitochondrial pathway, whereas Bcl-xS and Bax are positive regulators or inducers of this pathway [37]. c-FLIP is a caspase-8–like molecule that lacks the enzymatic residue to become a functional caspase [38]. c-FLIP inhibits caspase-8 activation by binding to caspase-8 or adapter molecules containing the death effector domain, such as FADD [38]. The anti-apoptotic proteins c-IAP1, c-IAP2, and XIAP are inhibitors of caspase-9, caspase-7, and caspase-3 [39–42]. Based on our immunoblotting analysis, c-FLIP is slightly up-regulated by 0.5-fold by 24 hr. Bcl-2 is slightly down-regulated after 24 hr. We also

observed that Bcl-xL displayed a biphasic effect, where it was up-regulated at the 6th hr and slightly reduced by 24 hr. However, Bcl-xS continued to rise over the 24-hr period. The protein level of c-IAP1 rose slightly after 6 and 12 hr but decreased back to basal level at 24 hr. c-IAP2 remained relatively high and constant

throughout the Act D treatment. The most pronounced effect was the down-regulation of XIAP. XIAP was reduced by twofold in 6 hr and by 24 hr XIAP was reduced to 1/10 of its original level. Immediate down-regulation of XIAP by 6 hr was also observed in Act D-sensitized DU-145 and PC-3 cells (data not shown).
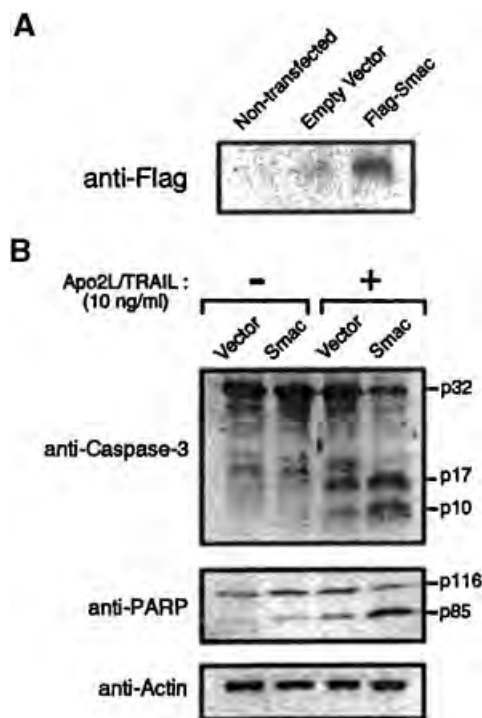
Overexpression of Smac/DIABLO Enhanced the Apo2L/TRAIL-Mediated Apoptosis in Prostate Tumor Cells Based on Caspase Activation

We investigated the role of XIAP in contributing to the tumor resistance to Apo2L/TRAIL in prostate tumor cells. We generated cells that overexpress Smac/DIABLO, which is a known neutralizing inhibitor of XIAP [43,44]. The transient transfectants were generated with a cDNA encoding the full-length Smac/DIABLO (pcDNA3.1-Smac/DIABLO), attached with a FLAG tag at the C terminus of the protein. The protein level of Smac/DIABLO in the transfectants was determined by the immunoblotting analysis against the flag tag to ensure the pcDNA3-Smac/DIABLO transfectants were overexpressing the protein. Indeed, the Smac/DIABLO transfectants expressed more Smac/DIABLO protein than the untreated or Act D–treated cells (Fig. 6A).

The level of caspase activation induced by Apo2L/TRAIL was examined in the untransfected cells and Smac/DIABLO transfectants. As shown in Figure 6B, the transfection process itself did not induce any caspase-dependent apoptotic events. Upon 24-hr treatment with Apo2L/TRAIL, some activation of caspase-3 was observed in vector-transfected cells, and this was also observed in cells transfected with Smac/DIABLO [44]; however, a higher level of caspase-3 activation and cleavage of PARP, which is a substrate of caspase-3, were seen in the Smac/DIABLO-transfected cells (Fig. 6B). The Smac/DIABLO-transfected cells were also sensitive to TRAIL-mediated apoptosis. These findings demonstrate that the expression of XIAP in CL-1 prostate tumor cells regulates the sensitivity to TRAIL and inhibition of XIAP by Smac/DIABLO in the transfected cells sensitized the cells to apoptosis by TRAIL. These findings also corroborate the role of XIAP down-regulation by Act D in the sensitization of the tumor cells to TRAIL apoptosis.

DISCUSSION

Our study shows that the hormone-independent prostate tumor cell lines (CL-1, DU-145, and PC-3) cells are resistant to Apo2L/TRAIL-mediated apoptosis and the resistance can be reversed by treatment with Act D. We characterized the molecular events in the Apo2L/TRAIL-mediated apoptosis pathways (caspase activation and mitochondrial activities) induced by Apo2L/

Fig. 6. Overexpression of Smac/DIABLO sensitizes resistant tumor cells to tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL) -mediated apoptosis. **A**: Overexpression of Smac/DIABLO in tumor cells transfected with pcDNA3-flag-Smac/DIABLO. Protein lysates were prepared from the untreated cells, cells transfected with pcDNA3 (empty vector), or pcDNA-flag-Smac/DIABLO. The expression of Smac/DIABLO was detected in an immunoblotting analysis by using the anti-flag mouse monoclonal antibody (M2). **B**: Apo2L/TRAIL-induced caspase-3 activity was enhanced in cells transfected with Smac/DIABLO. The untreated cells and cells overexpressing flag-Smac/DIABLO were treated with Apo2/TRAIL (10 ng/ml). Protein lysates were prepared and subjected to immunoblotting analysis for caspase-3 activity and poly-ADP-ribose-polymerase (PARP) cleavage. Caspase-3 activity is indicated by the appearance of active subunits (p17 and p10) and reduction of the pro-form (p32). The cleavage of PARP is shown by the disappearance of the whole protein (p116) and the appearance of the cleaved product (p85). The level of B-actin was determined to ensure equal loading of protein samples.

TRAIL alone, Act D alone, or Apo2L/TRAIL combined with Act D. Caspase-8, -9, and -3 were synergistically activated by the combination treatment with Apo2L/TRAIL and Act D, whereas the treatment with only Apo2L/TRAIL or only Act D did not activate detectable levels of these caspases. Although Apo2L/TRAIL was unable to induce apoptosis in prostate tumor cells by itself alone, Apo2L/TRAIL induced a significant amount of cytochrome c release and loss of mitochondrial membrane potential as early as 3 hr after the treatment. Because significant cytochrome c release and caspase activation triggered by the combination treatment were observed after 6 hr, it is likely that the

sensitizing effects of Act D had been initiated before this time. The regulatory effects of Act D on the protein expression of Apo2L/TRAIL receptors and various anti-apoptotic proteins were examined. The earliest changes observed at the 6th hr were the up-regulation of Bcl-xL and -xS proteins and down-regulation of XIAP, whereas other anti-apoptotic proteins were variably affected at later time points. However, the increase of Bcl-xL level after 6 hr might not be critical for the sensitization to Apo2L/TRAIL-mediated apoptosis, because it did not prevent the release of cytochrome c at that time. Inhibition of XIAP expression by transfection with Smac/DIABLO sensitized the cells to TRAIL apoptosis corroborating the role of Act D in sensitization. Therefore, Act-D–induced down-regulation of XIAP is in large part responsible for the sensitization of resistant prostate tumor cells to Apo2L/TRAIL-mediated apoptosis and the enhanced sensitivity to Apo2L/TRAIL is a result of complementary intracellular signalings mediated by both Apo2L/TRAIL and Act D.

The present findings provide novel information relevant to the mechanism of Apo2L/TRAIL resistance in prostate tumor cells. Apo2L/TRAIL could induce mitochondrial apoptotic events (the release of cytochrome c and loss of mitochondrial membrane potential) but could not effectively induce subsequent activation of caspases (caspase-9 and -3). Our data indicate that CL-1 is a type-II cell [14,15], because we could not observe significant level of active caspase-8, but it was sufficient to result in the mitochondrial activation. Currently, it is not known completely how caspase-8 is negatively regulated in type II cells. One possible negative regulator is cFLIP, which lacks the enzymatic domain of caspase-8 but is able to reduce formation of DISC by competitive DED domain binding with FADD and/or caspase-8. CL-1 cells clearly also express high level of cFLIP. The inability of cytoplasmic cytochrome c to initiate the activation of downstream caspases indicates that the resistance to Apo2L/TRAIL occurs downstream of the mitochondrial signaling. This observation points to the IAP family of proteins, the last line of apoptosis guardian, and most likely IAPs are the ones that confer the resistance to Apo2L/TRAIL in prostate tumor cells. These IAP family inhibitors exert their anti-apoptotic functions downstream of cytochrome c release by directly binding to caspases and preventing activation of effector caspases [40]. XIAP, among all the IAP proteins, is the most potent anti-apoptotic inhibitor [40]. When we examined the anti-apoptotic protein expression in the sensitized prostate tumor cells, XIAP was one of the earliest and the most affected anti-apoptotic proteins suppressed by Act D, whereas other anti-apoptotic factors were marginally affected at later

time points. In addition to XIAP, Bcl-xL/-xS were also up-regulated by Act D in 6 hr. However, these changes were not sufficient to block the mitochondrial apoptotic pathway triggered by the Apo2L/TRAIL plus Act D combination treatment, because we observed significant mitochondrial events at this time. Recent reports also have documented that Bcl-2 failed to block the Apo2L/TRAIL-induced release of cytochrome c and apoptosis [17,41]. Therefore, we think that Bcl-2 family members may not contribute significantly to the Apo2/TRAIL resistance in our tumor system.

The importance of XIAP in cancer resistance is further supported by a recent report documented that the expression of XIAP antisense cDNA down-regulates XIAP proteins and induces apoptosis directly in p53-positive cells [42]. The functional significance of IAP family proteins in prostate tumor cells is also confirmed by our findings that demonstrate that the overexpression of Smac/DIABLO in resistant prostate tumor cells enhances the Apo2L/TRAIL-mediated apptosis in the absence of Act D (Fig. 7. Smac/DIABLO is identified recently as a direct inactivator of IAP family proteins and has been shown to alleviate inhibition of the caspase activation cascade when it is released from mitochondria [43,44]. In prostate tumor cells, overexpression of Smac/DIABLO also down-regulates the protein level of XIAP like Act D [45]. Thus, based on these findings, XIAP might be a major contributing factor in Apo2L/TRAIL-resistance in prostate tumor cells.

Our findings with Apo2L/TRAIL resistance and sensitizing effects of Act D on prostate tumor cells are different from those observed in other tumors. Previous reports with transformed keratinocytes and breast tumor cells have documented that Apo2L/TRAIL resistance involves the loss of the agonist Apo2L/TRAIL receptors (DR4, DR5) or the overexpression of the decoy receptors (DcR1, DcR2) [28,34,36]. This was not the case for prostate tumor cells as they express sufficient agonist receptors to result in further mitochondrial apoptotic events. In addition, the reversal of Apo2L/TRAIL resistance by other genotoxic chemotherapeutic drugs in transformed keratinocytes, breast carcinoma, glioma, and colon carcinoma involves a p53-dependent induction of the agonist receptor DR5 [25,26,34,36]. In all three prostate tumor cell lines, functional wild-type p53 was not normally expressed and was not induced by Act D [46]. Our results show that the agonist Apo2L/TRAIL receptor DR4/TRAIL-R1 and the decoy receptor DcR1/TRAIL-R3 were gradually down-regulated over a 24-hr course, whereas DR5/TRAIL-R2, and DcR2/TRAIL-R4 receptors were affected minimally. Act D could not up-regulate the expression of the agonist receptors by means of a p53-dependent mechanism, and the changes in the receptor
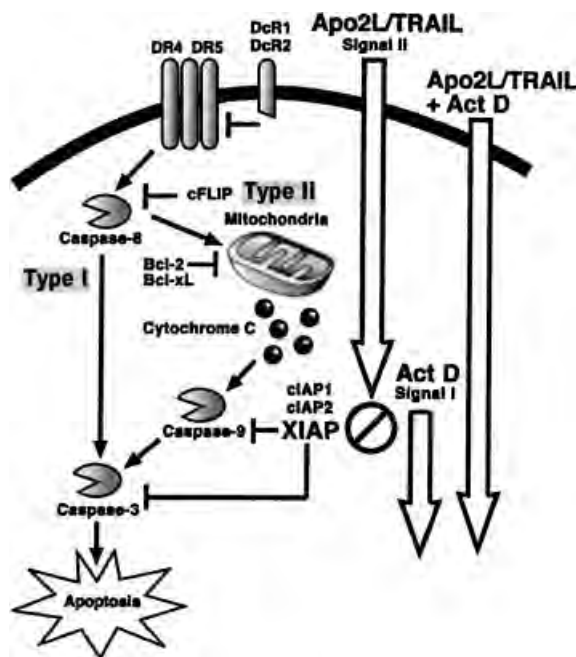
Fig. 7. Proposed model of actinomycin D (Act D)-sensitized tumor necrosis factor–related apoptosis-inducing ligand (Apo2L/TRAIL)-mediated apoptosis. Apo2L/TRAIL initiates apoptosis by trimerization of its cognate receptors DR4 and DR5, followed by activation of the initiator caspase-8. Active caspase-8 can directly activate caspase-3 or indirectly activate caspase-9 by means of the mitochondrial pathway. The direct pathway that activates capase-3 after caspase-8 activation is known as the type I pathway; the indirect activation of caspase-3 by means of the activation of caspase-9 is termed the type II pathway (described in Scaffidi et al. [14]). In the type II pathway, caspase-9 is activated when cytochrome c is released from the mitochondria, which is triggered by active caspase-8. The activation of caspase-3 leads to the final apoptotic phenotypes such as DNA fragmentation and chromosomal condensation. Various anti-apoptotic proteins inhibit each signaling event throughout the pathway. c-FLIP inhibits the activation of caspase-8. Bcl-2–related family proteins guard against mitochondrial release of cytochrome c. IAP family proteins directly inhibit the activation of caspase-9 and -3. Apo2L/TRAIL could induce cytochrome c but did not activate caspase-9 (Signal II). The blockade of signal I is potentially mediated primarily through XIAP (stop sign). Pretreatment with Act D suppresses the expression of XIAP, thus removing the block (Signal I). The combination treatment of Apo2L/TRAIL and Act D then leads to completion of the apoptotic pathway.

protein expression (DR4/TRAIL-R1 and DcR1/TRAIL-R3) most likely did not affect the death signal that has already initiated very early on. Recent reports also documented that Act D could sensitize to Apo2L/TRAIL-mediated apoptosis by down-regulation of c-FLIP in neuroblastoma cells [47]. However, in our case, c-FLIP was up-regulated at 12 and 24 hr. These changes might not have blocked the initial processing of caspase-8 because Apo2L/TRAIL have already

triggered the mitochondrial pathway at the beginning. Our finding that XIAP is down-regulated by Act D is also corroborated by the same report on neuroblastoma cells where XIAP was also shown to be down-regulated, although at a later time [47].

This study also highlights the unique sensitizing role of Act D as an agent that may down-regulates selectively a subset of gene products to sensitize Apo2L/TRAIL-mediated killing. Act D is commonly known as an antibiotic and anti-tumor agent, and it exerts its anti-tumor activity through inhibition of transcription [48,49]. It has been shown as a direct inducer of apoptosis that activates both caspase-dependent and independent pathways [50]. In our study, the prostate tumor cell lines are resistant to Act D–mediated killing, yet Act D sensitizes the tumor cells to Apo2L/TRAIL-mediated apoptosis. A synergistic cytotoxicity was achieved by nontoxic concentrations of Act D and Apo2L/TRAIL. Our analyses revealed that Act D could exert a preferential down-regulation of XIAP in prostate tumor cells over other anti-apoptotic proteins at early time points. Similar selective down-regulation of an anti-apoptotic protein (Bcl-xL) by Act D was also observed in an earlier study in AIDS-Kaposi's sarcoma from our laboratory [23]. Thus, Act D may modulate different proteins in different cell systems.

The mechanism by which Act D down-regulates XIAP expression is not known. It may possibly involve selective inhibition of XIAP gene transcription. The selectivity may be derived from the DNA-binding property of Act D in a sequence-specific manner. Act D potentially binds DNA and interferes with the docking of certain transcriptional factors to DNA [51,52]. Another possible explanation for the decreased expression of XIAP is translational interference and rapid turnover of XIAP. Recent reports have demonstrated that translation of XIAP is controlled by a specific translational regulatory mechanism [53]. Act D potentially can interfere with this translation regulatory program. In addition, several recent reports have shown that cleavage or degradation of XIAP is an important step in the amplification of apoptotic signals [54,55]. XIAP can also even catalyze its own ubiquitination and protein degradation in response to various apoptotic stimuli [56]. Although Act D was unable to induce apoptosis in prostate tumor cells, it might still retain its ability to induce degradation of XIAP. Further studies are needed to elucidate whether Act D selectively down-regulates XIAP at the level of mRNA or protein.

Based on our results, we propose the following two-signal model for Apo2L/TRAIL-mediated apoptosis of CL-1 cells sensitized by Act D (Fig. 7). The model describes the synergistic induction of apoptosis caused by the complementary pro-apoptotic effects of Act D and Apo2L/TRAIL. Apo2L/TRAIL provides the

necessary apoptotic-triggering signal (signal II, Fig. 7). The death signal triggered by Apo2L/TRAIL proceeds down to the mitochondrial apoptotic events (release of cytochrome c and loss of mitochondrial membrane potential), but it is not sufficient to complete a full caspase activation. To allow the Apo2L/TRAIL-induced death signal to proceed further, a modifying signal (signal I, Fig. 7) is required to complete the full Apo2L/TRAIL apoptotic signaling and reverse the resistance. Act D provides the modifier signal (signal I) by potentially down-regulating XIAP and/or other unidentified protective factors. The down-regulation of XIAP, thus, provides a potential method to complement the Apo2L/TRAIL signaling where it is primarily inhibited—the effector caspases—to induce high level of apoptosis.

The proposed model illustrates a unique situation under which resistant cancer cells can be rendered sensitive to immune-mediated apoptosis, that is, the two signals must complement each other well in their pro-apoptotic effects. In our previous study, we documented that Act D alone also sensitized the prostate tumor cells to TNF-α and Fas-mediated apoptosis [31]. Thus, resistance to immune mechanisms of cytotoxicity can be overcome by sensitization. In our system, the selective suppression of XIAP protein expression by Act D and the activation of mitochondrial events by Apo2L/TRAIL have been implicated as the key steps for the complementation of Act D and Apo2L/TRAIL to occur in a type II apoptotic pathway. Therefore, we predict that other agents that mimic the sensitizing activity of Act D can also complement Apo2L/TRAIL to induce apoptosis in resistant tumor cells. Indeed, both *cis*-diaminedichloroplatinum (II), cisplatin and Adriamycin™ sensitized the tumor cells to Apo2L/TRAIL apoptosis [31].

Apo2L/TRAIL has been postulated to be an effective anti-cancer agent, because it is believed that Apo2L/TRAIL selectively kills cancer cells and spares normal ones. Contrary to this accepted notion, a recent report has shown that normal prostate epithelial cells are actually sensitive to Apo2L/TRAIL-mediated killing and the killing could be enhanced by the protein synthesis inhibitor cycloheximide [57]. It is possible that treatment with the combination of Act D and Apo2L/TRAIL could lead to significant cell death in normal prostate epithelial cells. The killing may be advantageous to remove any normal prostate cells that may later develop into cancer or benign hyperplasia.

Today, resistance of tumor to conventional therapies remains a major problem. To circumvent this problem, the complementation model proposed here can serve as a guideline to search for agents that can enhance the therapeutic potential of preexisting or novel therapeutics such as Apo2L/TRAIL against immune-resistant

or drug-resistant prostate cancer. Thus, our studies document the need for complementation of immunotherapy protocols by combination with agents that selectively sensitize the tumor cells to the cytotoxic lymphocytes [58]. Because it is not clear what dictates the preferential signaling of particular cytotoxic mechanisms by anti-tumor cytotoxic lymphocytes, it is likely that, due to the heterogeneity of the tumor cells population, there exists also heterogeneity in their sensitivity to particular cytotoxic mechanisms. The immunosensitization described herein provides a novel approach to override immunoresistance and should potentiate the immunotherapy response.

ACKNOWLEDGMENTS

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X-linked Inhibitor of Apoptosis (XIAP) Blocks Apo2 Ligand/Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis of Prostate Cancer Cells in the Presence of Mitochondrial Activation: Sensitization by Overexpression of Second Mitochondria-derived Activator of Caspase/Direct IAP-binding Protein with Low pI (Smac/DIABLO)

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Abstract

The resistance to Apo2 ligand (Apo2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis could be overcome by treatment with subtoxic concentrations of actinomycin D (Act D) in prostate tumor cells. Furthermore, the sensitization to Apo2L/TRAIL-mediated apoptosis by Act D positively correlated with selective down-regulation of X-linked inhibitor of apoptosis (XIAP). In this study, we examined whether second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a known inhibitor of apoptosis (IAP)-neutralizing protein, sensitizes resistant prostate tumor cells to Apo2L/TRAIL-mediated apoptosis. The prostate tumor cell line CL-1 was treated with Apo2L/TRAIL, Act D, or a combination of the two. The apoptosis-mediated signaling pathway was examined by Western blotting and flow cytometry. Furthermore, CL-1 cells transfected with the anti-IAP inhibitor Smac/DIABLO were examined for sensitivity to Apo2L/TRAIL. Whereas Apo2L/TRAIL induced the release of cytochrome *c* and endogenous Smac/DIABLO in the CL-1 tumor cells, the cytosolic levels of both molecules were not sufficient to induce apoptosis. Transient transfectants with a Smac/DIABLO cDNA encoding a neutralizing inhibitor of IAPs were sensitized to Apo2L/TRAIL-mediated apoptosis. The sensitization to Apo2L/TRAIL by Smac/

DIABLO overexpression was a result of synergistic activation of caspases-3, -9, and -8. Treatment of the Smac/DIABLO transient transfectant with Apo2L/TRAIL enhanced the release of Smac/DIABLO from mitochondria and led to reduction of IAP family proteins (XIAP, c-IAP1, and c-IAP2). These results show that Smac/DIABLO can sensitize CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis. Thus, up-regulation of Smac/DIABLO and sensitization to Apo2L/TRAIL-mediated apoptosis are of potential clinical application in the immunotherapy of drug-/Apo2L/TRAIL-resistant tumors.

Introduction

The TNF[3] ligand superfamily plays an important role in the host immune defense against cancer as an antitumor death-inducing agent (1). The TNF ligand members induce tumor programmed cell death or apoptosis by binding to their cognate death receptors on the cell surface (2–6). Recombinant TNF ligands have been widely explored as potential therapeutic agents against several types of cancer (7). Among the current members, Apo2L/TRAIL is the most promising for therapeutic use because it has been shown to kill a wide variety of malignant tumors while eliciting little systemic toxicity in experimental animal models (8, 9).

Similar to TNF-α and Fas, Apo2L/TRAIL induces apoptosis in sensitive tumor target cells by the death receptor pathway (9, 10). Upon cross-linking with the death receptors TRAIL-R1/DR4 and/or TRAIL-R2/DR5, Apo2L/TRAIL initiates aggregation of the death receptors, recruitment of the adaptor molecule Fas-associated death domain (FADD), and activation of initiator caspase-8 (11–13). The active caspase-8 or caspase-10 triggers a caspase activation cascade by directly activating effector caspase-3 (type I pathway) or by diverting

[3] The abbreviations used are: TNF, tumor necrosis factor; Ab, antibody; Act D, actinomycin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GFP, green fluorescence protein; IAP, inhibitor of apoptosis; MTS, mitochondrial targeting sequence; PARP, poly(ADP-ribose) polymerase; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; XIAP, X-linked inhibitor of apoptosis; Apo2L, Apo2 ligand; FBS, fetal bovine serum; 7-AAD, 7-amino-actinomycin D.

the death signal to mitochondria (type II pathway; Refs. 14–16). The type II pathway, which involves the release of cytochrome *c* from mitochondria, further amplifies the death signal by activation of caspase-9 and ensuing activation of caspase-3 (15–18).

The Apo2L/TRAIL apoptotic signaling pathway is subjected to several levels of inhibitory regulation. These include surface expression of two decoy receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2; Ref. 10); FLIP, a dominant negative form of caspase-8 that lacks the caspase catalytic site (19, 20); and, at the mitochondrial level, Bcl-2 antiapoptotic members (Bcl-2, Bcl-xL, Bfl-1/A1, and Mcl-1; Ref. 21). Finally, at the caspase level, IAP family members (c-IAP1, c-IAP2, XIAP, and survivin) bind to caspases-9, -3, and -7 and prevent the onset of the caspase activation cascade (22–24). All of these antiapoptotic molecules render cells insensitive to various apoptotic stimuli, including Apo2L/TRAIL. Their overexpression has been shown to be associated with tumor resistance to apoptosis-inducing tumor therapies (25).

One of the major problems in cancer treatment today is the development or acquisition of tumor resistance (25). Because many conventional cancer therapies such as chemotherapy and radiation eradicate tumors by apoptosis, high expression of antiapoptotic molecules will render tumor cells resistant to the conventional therapies. However, novel therapeutics may also use apoptosis to eradicate tumors. Experimental immunotherapeutic approaches based on immune cytotoxic molecules such as Apo2L/TRAIL will also most likely be ineffective against chemoresistant tumor cells. Therefore, the tumor resistance to apoptosis must be reversed to enhance the efficacy of cancer therapy.

To overcome tumor resistance to Apo2L/TRAIL, we have used Act D to sensitize immunoresistant prostate tumor cells to Apo2L/TRAIL-mediated apoptosis (26). We have also shown that sensitization to Apo2L/TRAIL killing is associated with the preferential down-regulation of XIAP (27). Recently, a mitochondrial molecule named Smac/DIABLO has been documented to be a neutralizing inhibitor of the apoptotic inhibitor IAP family proteins (28, 29). Upon receiving a death signal, mitochondria release Smac/DIABLO into the cytoplasm, in addition to the release of cytochrome *c* (28, 29). Cytoplasmic Smac/DIABLO binds to IAP family members and relieves the IAP-mediated inhibition of caspases-9 and -3 (28–30). In this study, we examined whether the overexpression of Smac/DIABLO, which down-regulates the level of XIAP, sensitizes CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis. This study also compared the apoptotic signaling events induced by Act D, Smac/DIABLO overexpression, and Apo2L/TRAIL.

Materials and Methods

Cells and Reagents. The immunoresistant prostate tumor cell line CL-1 was kindly provided by Dr. Arie Belldegrun (University of California Los Angeles). The establishment and characterization of CL-1 cells have been described previously (31). The prostate cells were cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (Gemini Bioproducts, Woodland, CA). Act D was purchased

from Sigma (St. Louis, MO). Recombinant human Apo2L/TRAIL was purchased from Peprotech (Rocky Hill, NJ).

Constructs. The construct pcDNA-FLAG-smac overexpressing Smac/DIABLO was kindly provided by Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX). The production of the expression construct was described by Du *et al.* (28). The control vector pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA). The pEGFP-N1 expression construct encoding GFP was purchased from Clontech (Palo Alto, CA).

Abs. The monoclonal anti-FLAG Ab M2 clone was purchased from Sigma. The polyclonal anti-Smac/DIABLO Ab was kindly provided by Dr. Xiaodong Wang. The polyclonal anti-survivin Ab was purchased from Pro-Sci (San Diego, CA). The monoclonal anti-actin Ab was purchased from Chemicon (Temecula, CA). The polyclonal Abs against IAP family proteins (c-IAP1, c-IAP2, and XIAP) were purchased from Trevigen (Gaithersburg, MD). The anti-Bcl-2 monoclonal Abs and anti-c-FLIP Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Upstate Biotechnology (Lake Placid, NY), respectively. Monoclonal anti-cytochrome *c* Ab was purchased from BD PharMingen (San Diego, CA).

Propidium Iodide-based DNA Fragmentation Assay by Flow Cytometry. The propidium iodide-based flow cytometric DNA fragmentation assay was adopted from Nicoletti *et al.* (32). Approximately 2×10^5 prostate tumor cells were seeded in each well in 12-well plates in RPMI 1640 supplemented with 10% FBS overnight. Recombinant Apo2L/TRAIL (10 ng/ml) and Act D (100 ng/ml) were simultaneously added to the cell cultures, and the samples were collected at various time periods after the addition of Act D and Apo2L/TRAIL. Measurement of DNA fragmentation in propidium iodide-stained cells was performed using an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). Region markers were drawn for sub-G_1, G_0-G_1, S, and G_2-M populations for quantitation of the cell populations by the flow cytometer. The sub-G_1 population represents the cells containing DNA hypoploidy, a characteristic of apoptotic cells undergoing DNA fragmentation.

Dual-Color Fluorescence Analysis of Apoptotic Cells by Flow Cytometry. The dual-color fluorescence analysis of apoptotic cells by flow cytometry was modified from the original method. Briefly, the CL-1 prostate tumor cells were previously transfected with pEGFP-N1, followed by 24 h of humidified incubation at 37°C for the expression of GFP. The next day, the cells were treated with or without recombinant Apo2L/TRAIL molecules for additional 24-h incubation. After 48 h, the cells were harvested using PBS supplemented with 0.5 mM EDTA. The detached cells were checked under a fluorescence microscope to ensure expression of GFP in the cells. The cells were then centrifuged down and washed once with cold PBS, followed by resuspension of the cells in cold 0.5 ml of 20 μg/ml 7-AAD solution in PBS in each tube. The cells were incubated at 4°C for 20 min. After the incubation, the samples were analyzed on an Epics XL flow cytometer (Coulter Electronics, Inc.). The fluorescence of GFP was analyzed in the FL1 channel (green fluorescence). The green-positive cells were gated and further analyzed in
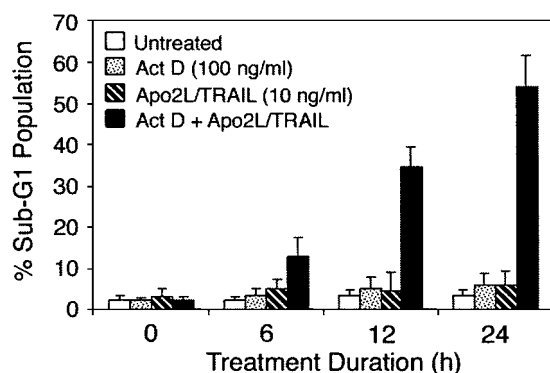
Fig. 1. Synergistic induction of apoptosis in prostate tumor CL-1 cells by combination treatment with Apo2L/TRAIL and Act D. The cells were pretreated with or without Act D (100 ng/ml) for 6 h, followed by the addition of Apo2L/TRAIL (10 ng/ml) for the various times indicated (6, 12, and 24 h), or left untreated. The percentages of apoptotic cells were determined by a flow cytometric cell cycle analysis based on propidium iodide staining (see "Materials and Method"). The *bars* represent the percentages of sub-G_1 populations $\pm$ SD ($n = 3$) at various incubation periods with Apo2L/TRAIL. The cells were harvested and fixed at different time intervals, and then all samples were accumulated and quantitated in a flow cytometer concurrently.

the FL3 channel (red fluorescence). The apoptotic cells are more permeable to 7-AAD, and thus they appear to be more fluorescent in red and can be distinguished from the live cells.

Transient Transfection of Tumor Cells. The transfection of CL-1 tumor cells was performed using the polycationic liposome reagent LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). The transfection was done according to the manufacturer's instructions. The vector DNA and pcDNA3-Smac/DIABLO were first mixed with the liposome reagent in a ratio of 2.0 μl of LipofectAMINE 2000:1.25 μg of DNA in 0.5 ml of serum-free RMPI 1640 (Mediatech) for 20 min at room temperature. The 0.5-ml liposome-DNA mixture was then added to each well of cells plated on 24-well plates for transfection. After 6 h, the transfection medium was removed, and fresh medium containing 10% FBS was added to allow the recovery of the cells.

Immunoblotting. This was determined as we described previously (27).

Isolation of the Cytosolic Fraction and Determination of the Release of Cytochrome *c* and Smac/DIABLO. CL-1 tumor cells were grown to near confluence (approximately 8.0×10^6 cells) in 100×20-mm culture dishes. After the treatments, both the floating and attached cells were collected. The cell pellets were washed twice in cold PBS and resuspended in 2 volumes of homogenization buffer [0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and a tablet of Mini complete protease inhibitor mixture (Roche, Indianapolis, IN) per 10 ml of homogenization buffer]. Cells were incubated on ice for 30 min to let the cells swell and lyse. Cell homogenates were prepared by disrupting the cells with 40 strokes in a 2-ml Dounce glass homogenizer with a loose-size pestle. Unbroken cells and
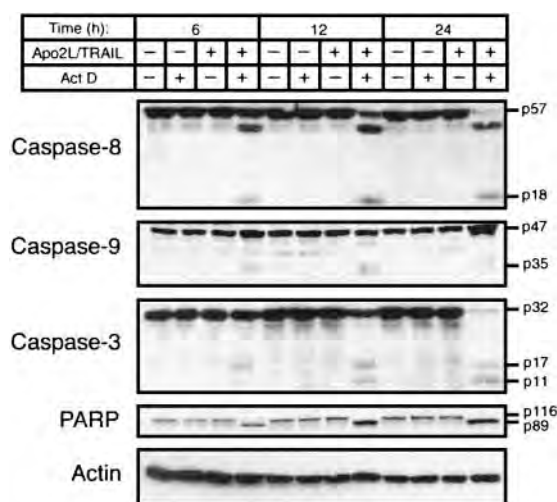
Fig. 2. Apo2L/TRAIL-induced caspase activation in Act D-treated CL-1 cells. The cells were pretreated with Act D (100 ng/ml) for 6 h and then treated with Apo2L/TRAIL (10 ng/ml) for the different incubation periods indicated (6, 12, and 24 h), and lysates were collected at those times for immunoblotting analysis. A panel of polyclonal Abs detected the following caspase proteins and PARP: caspase-8 proform (*p57*) and its cleaved activated subunit (*p18*); zymogen pro-caspase-9 (*p47*) and cleaved activated intermediate (*p35*); zymogen pro-caspase-3 (*p32*) and cleaved activated subunits (*p17* and *p11*); and intact PARP protein (*p116*) and PARP cleavage product (*p89*). β-Actin was also detected to ensure equal loading of the samples. Total protein extracts were separated on a 15% polyacrylamide gel for the detection of PARP and caspase cleavage. Thus, the migration of PARP is much smaller (M_r 166,000 to M_r 89,000) compared with that of caspase-3 (M_r 32,000 to M_r 11,000). The PARP bands are located near the top of the gel, whereas caspases ran much further down.

nuclei were centrifuged down ($2,500 \times g$, 4°C, 5 min) twice. Supernatants, which contained mitochondria, were collected and subjected to further centrifugation at $16,000 \times g$ at 4°C for 30 min to pellet down the mitochondrial fraction. The postmitochondrial supernatants were successively filtered through 0.2 and 0.1 μm Ultrafree MC filters (Millipore, Bedford, MA) to yield a cytosolic fraction. The cytosolic fraction was then subjected to Western blotting analysis for cytochrome *c* and Smac/DIABLO.

Statistical Analysis. All quantitative assays were set up in triplicates, and the results were expressed as the mean $\pm$ SD. Statistical significance for comparison between two groups of data was determined using Student's *t* test. Significant differences were considered to exist for those probabilities <5% ($P < 0.05$).

Results

Pretreatment with Act D Sensitizes CL-1 Tumor Cells to Apo2L/TRAIL-mediated Apoptosis. Titration of both TRAIL and Act D for optimal cytotoxicity revealed that 100 and 10 ng/ml Act D were the most optimal concentrations for synergistic killing of the androgen-independent CL-1 prostate tumor cells (27). The cells were pretreated with Act D (100 ng/ml) for 6 h, followed by the addition of human recombinant Apo2L/TRAIL (10 ng/ml). The cells were sampled at various time points during the Apo2L/TRAIL treatment (6, 12, and 24 h) to assess apoptosis. The combination treat-
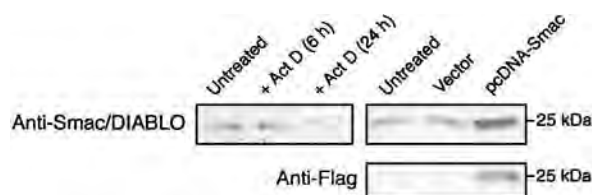
Fig. 3. Expression of Smac/DIABLO in prostate tumor cells. The level of endogenous Smac/DIABLO mature protein (M_r 25,000) was determined in untreated cells, Act D-treated cells, and cells transfected with pcDNA3 (control vector) or pcDNA-FLAG-Smac/DIABLO using a polyclonal anti-Smac/DIABLO Ab in immunoblotting analysis. The duration of Act D treatment is indicated (6 or 24 h). The expression of FLAG-tagged Smac/DIABLO in the transfectants was detected using a monoclonal anti-FLAG Ab.

ment resulted in significant enhancement of apoptosis with up to 55.0% of apoptotic cells displaying DNA hypoploidy, whereas treatment with Act D or Apo2L/TRAIL alone resulted in little DNA fragmentation at the 24 h time point (Fig. 1).

Pretreatment with Act D, followed by Treatment with Apo2L/TRAIL, Activates Caspases-8, -9, and -3. The synergistic apoptotic killing by Act D and Apo2L/TRAIL determined by the propidium iodide flow cytometric DNA fragmentation assay was confirmed by immunoblotting analyses for caspase activation (Fig. 2). After pretreating the cells with Act D (100 ng/ml) for 6 h, followed by 6 h of treatment with Apo2L/TRAIL (10 ng/ml), noticeable levels of caspases-8, -9, and –3 and cleavage of PARP (a cellular substrate of caspase-3) were observed in the combination-treated cells. The levels of activated caspases and cleaved PARP products became more apparent at the later time points (12 and 24 h) of the Apo2L/TRAIL treatment (Fig. 2).

Overexpression of Smac/DIABLO Enhanced Apo2L/TRAIL-mediated Apoptosis in Prostate Tumor Cells Based on a 7-AAD Staining Assay. We examined whether the overexpression of Smac/DIABLO, a neutralizing inhibitor of IAPs, could enhance Apo2L/TRAIL-mediated killing. The transient transfectants were generated with a cDNA encoding the full-length Smac/DIABLO (pcDNA3.1-Smac/DIABLO), attached with a FLAG tag at the COOH terminus of the protein. The protein level of Smac/DIABLO in the transfectants was determined by immunoblotting analysis to ensure that the cells transfected with pcDNA3-Smac/DIABLO were overexpressing the protein (Fig. 3). The level of mature Smac/DIABLO in the transfectants was also compared with that in untreated and Act D-treated cells. Indeed, the Smac/DIABLO transfectants expressed more Smac/DIABLO protein than the untreated or Act D-treated cells (Fig. 3).

In the following flow cytometry-based apoptosis assay, an expression construct encoding GFP was cotransfected at a 1:5 ratio with Smac/DIABLO or control constructs to track the Smac/DIABLO transfectants. The transfection efficiency was between 13% and 34%, as measured by flow cytometry (data not shown). After treatment with Apo2L/TRAIL, we further measured the level of apoptosis in the GFP-positive transfectants by 7-AAD staining. The amount of apoptosis is reflected in the increased staining of the semipermeable red fluorescent dye 7-AAD (33, 34). In a log-red fluorescence
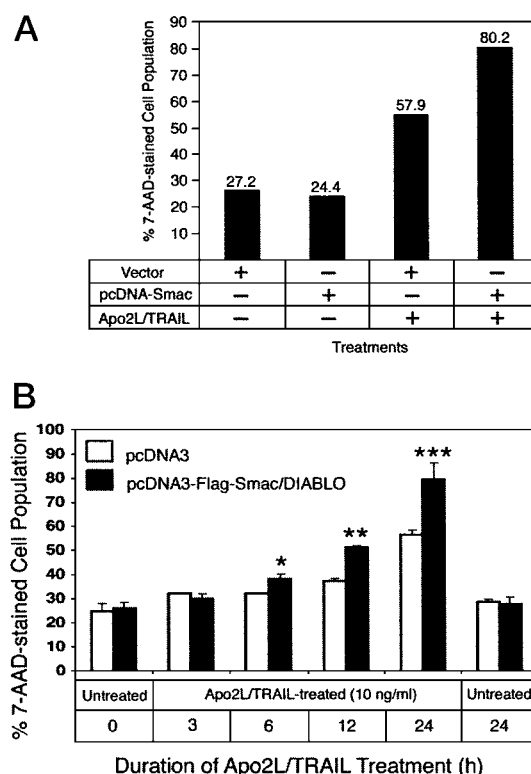
Fig. 4. Synergistic induction of apoptosis in prostate tumor CL-1 cells by overexpression of Smac/DIABLO and treatment with Apo2L/TRAIL. The cells were transfected with either the control vector (pcDNA3) or pcDNA3-FLAG-Smac/DIABLO and cotransfected with pEGFP-N1 to track GFP-expressing transfectants. The cells were incubated at 37°C for 24 h after transfection and then treated with recombinant human Apo2L/TRAIL (100 ng/ml) at various time points. The percentages of apoptotic cells were determined by a flow cytometric analysis based on 7-AAD uptake. To quantify the amount of apoptotic cells in the transfectants, the total GFP-expressing cell populations that display higher green fluorescence were gated and further analyzed for red fluorescence. The 7-AAD-stained cells are those cells that have undergone apoptosis and displayed a red fluorescent population in a forward scatter *versus* red fluorescence histogram. The percentage of 7-AAD-stained cells was gated and counted by flow cytometry. *A,* apoptotic effects of 24-h Apo2L/TRAIL treatment on Smac/DIABLO transfectants and control transfectants. The *X* axis indicates the treatment conditions [transfection with either control vector or pcDNA3-FLAG-Smac/DIABLO, followed by 24-h treatment with or without Apo2L/TRAIL (10 ng/ml)]. The *bars* represent the number of 7-AAD-stained cells (apoptotic cells). *B,* apoptotic effects of Apo2L/TRAIL treatment (10 ng/ml) on Smac/DIABLO transfectants and control transfectants at various time intervals. The *bars* represent the mean ± SD ($n = 3$) of the percentages of the cells stained with 7-AAD (the apoptotic cells). The time intervals (0, 3, 6, 12, and 24 h) at the *bottom* indicate the duration of Apo2L/TRAIL treatment. *Black bars* indicate Smac/DIABLO transfectants, and *white bars* indicate cells transfected with the control vector pcDNA3. Untreated transfectants at the 0 and 24 h time points were included for comparison. Statistical signficance: *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.05$.

versus forward scatter histogram, the late apoptotic cells displayed brighter 7-AAD red fluorescence and smaller cell size (forward scatter).

Using this flow cytometry-based protocol, we determined that the expression of Smac/DIABLO enhances Apo2L/TRAIL-mediated apoptosis. When the vector- or pcDNA-Smac/DIABLO-transfected cells were left untreated for 48 h after transfection, 27.2% and 24.4% of the total green cells
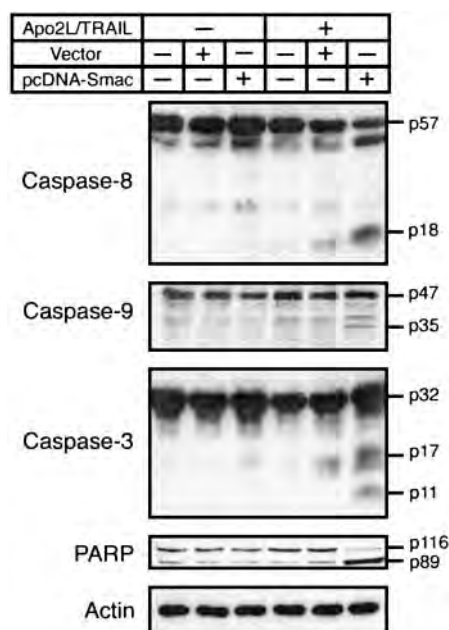
Fig. 5. Apo2L/TRAIL-induced caspase activation in Smac/DIABLO transfectant and control vector transfectant. The cells were transfected with pcDNA3-FLAG-Smac/DIABLO or control vector (pcDNA3) and then treated with Apo2L/TRAIL (10 ng/ml). After a 24-h treatment with Apo2L/TRAIL, lysates were collected for immunoblotting analysis. Detection of active caspase enzymatic subunits is similar to that in Fig. 2.

Fig. 6. Apo2L/TRAIL-induced release of cytochrome *c* and Smac/DIABLO from mitochondria. *A,* treatments with Apo2L/TRAIL (10 ng/ml) increased the level of cytochrome *c* and mature Smac/DIABLO in the cytoplasm in both the Act D-pretreated cells and untreated cells. Prostate tumor cells were pretreated with or without Act D for 6 h, followed by treatment with Apo2L/TRAIL for the various time periods indicated (6, 12, and 24 h). At each time point, cells were harvested, and cytosolic fractions were extracted. The cytosolic extract was later subjected to immunoblotting analysis. Cytochrome *c* and mature Smac/DIABlO were detected as M_r 15,000 and M_r 25,000 protein bands, respectively. *B,* treatments with Apo2L/TRAIL (10 ng/ml) increased the level of cytochrome *c* and mature Smac/DIABLO in the cytoplasm in all samples (nontransfectant, control vector transfectant, and Smac/DIABLO transfectant). Cytosolic fractions were isolated after a 24-h treatment with Apo2L/TRAIL (10 ng/ml) and later subjected to immunoblotting analysis for detection of cytochrome *c* and mature Smac/DIABLO. β-Actin was detected to ensure equal loading of protein samples.

underwent apoptosis, respectively (Fig. 4*A*). When the Smac/DIABLO-transfected cells were treated with Apo2L/TRAIL for 24 h after the 24-h transfection, the cells became more apoptotic (57.9% for the vector-transfected cells and 80.2% for the pcDNA-Smac/DIABLO transfected cells; Fig. 4*A*). The killing of transfectants by Apo2L/TRAIL was further examined at various time points (3, 6, 12, and 24 h). The increase in the percentage of apoptotic cells in the Smac/DIABLO-transfected cells was observed as early as 6 h after treatment (5% increase; Fig. 4*B*). At 24 h, maximally enhanced Apo2L-mediated killing by Smac/DIABLO overexpression was observed (22% increase).

Activation of Caspases by the Combination of Overexpression of Smac/DIABLO and Treatments with Recombinant Apo2L/TRAIL. The level of caspase activation was examined by the combination of Smac/DIABLO overexpression and Apo2L/TRAIL treatment. As shown in Fig. 5, the transfection process itself did not induce any detectable caspase-dependent apoptotic events. Upon 24-h treatment with Apo2L/TRAIL, slight activation of caspases-8 and -3 was observed in vector-transfected cells, and higher levels of caspases-8, -9, and -3 and cleavage of PARP, a substrate of caspase-3, were seen in the Smac/DIABLO-transfected cells (Fig. 5). Although Apo2L/TRAIL slightly activates caspase-3 in the vector-transfected cells, it was not sufficient to result in significant cleavage of PARP, compared with the untreated controls. Because Apo2L/TRAIL could activate a full apoptotic program involving caspases in the Smac/DIABLO-transfected cells, Smac/DIABLO is effective in sensitizing CL-1 tumor cells to Apo2L/TRAIL-mediated apoptosis.
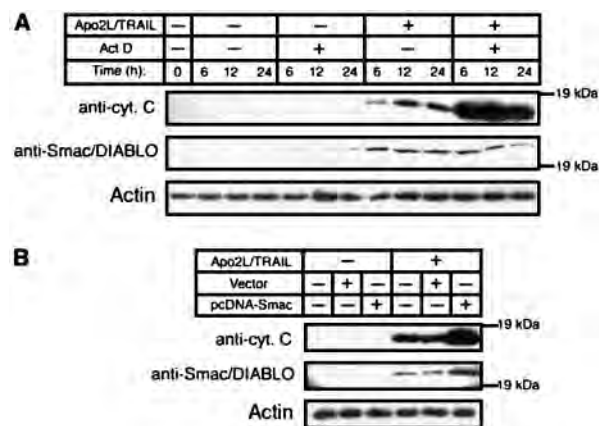
Apo2L/TRAIL Induces the Release of Cytochrome *c* and Smac/DIABLO in Untreated Cells, Act D-treated Cells, and Smac/DIABLO-transfected Cells. The synergistic activation of caspase-9 by Apo2L/TRAIL is clearly evident in both the Act D-sensitized cells and cells overexpressing Smac/DIABLO. The activation of caspase-9 relies on the formation of apoptosome induced by cytoplasmic cytochrome *c* and may also require Smac/DIABLO in the cytoplasm to counteract the IAP inhibition on caspase-9 (30). Thus, we compared the release of cytochrome *c* and Smac/DIABLO from the mitochondria induced by Apo2L/TRAIL in both Act D-treated cells and Smac/DIABLO transfectants. As shown in the *top panels* of Fig. 6, *A* and *B,* Apo2L/TRAIL alone induces the release of cytochrome *c* in all of the untreated, Act D-treated, and Smac/DIABLO-transfected cells. However, Apo2L/TRAIL can only activate caspases in Act D-treated cells and Smac/DIABLO transfectants, and not in untreated cells (Figs. 2 and 5), indicating that the release of cytochrome *c* induced by Apo2L/TRAIL alone is not sufficient to induce apoptosis.

Next, we examined the release of Smac/DIABLO from the mitochondria induced by Apo2L/TRAIL in both experimental systems (Fig. 6, *A* and *B, bottom panels*). Similar to the release of cytochrome *c,* Apo2L/TRAIL also induced the release of Smac/DIABLO from the mitochondria in all samples, including the untreated cells. However, Apo2L/TRAIL-induced release of Smac/DIABLO, in conjunction with the release of cytochrome *c,* was not sufficient to induce caspase activation and apoptosis (Figs. 2, 4, and 5). Only when the cells were pretreated with Act-D for 6 h or over-

expressed Smac/DIABLO were the cells sensitized to undergo Apo2L/TRAIL-mediated apoptosis. Upon stimulation with Apo2L/TRAIL, the Smac/DIABLO transfectants were able to release a higher level of Smac/DIABLO protein in comparison with the untreated or Act D-treated cells. The higher level of Smac/DIABLO in the cytoplasm might be responsible for the sensitization of Smac/DIABLO transfectants to Apo2L/TRAIL-mediated apoptosis. Thus, the result suggests that the endogenous level of Smac/DIABLO was not sufficient to render cells sensitive to Apo2L/TRAIL-mediated apoptosis and that increased expression of Smac/DIABLO might be necessary for inactivation of IAPs and sensitization to Apo2L/TRAIL-mediated apoptosis.

Protein Level of Various Antiapoptotic Molecules in Act-D-treated Cells and Smac/DIABLO-transfected Cells before and after Treatment with Apo2L/TRAIL. Previously, Smac/DIABLO has been shown to bind to the IAP family proteins (c-IAP1, c-IAP2, XIAP, and survivin) and relieves the inhibition of caspase-3 and -9 (28–30). The binding of Smac/DIABLO to IAPs will disable the direct binding of IAPs to active caspases. Once IAPs are free from caspase by Smac/DIABLO, they can be easily ubiquitinated and degraded by proteosome (35). The modulation of the protein levels of various antiapoptotic factors by Act D treatment and overexpression of Smac/DIABLO was compared. As seen in Fig. 7A, Act D selectively down-regulated the level of XIAP protein after 6 h of treatment, whereas other antiapoptotic proteins (c-IAP1, c-IAP2, survivin, FLIP, and Bcl-2) remained largely unaffected. On the other hand, the transfection with Smac/DIABLO cDNA did result in significant reduction of XIAP (Fig. 7A). In addition, the transfection with Smac/DIABLO cDNA and vector alone resulted in a slight reduction of c-IAP1 and a significant induction of c-IAP2 (Fig. 7A). These changes are most likely caused by the nonspecfic effects of liposomal transfection. The overexpression of Smac/DIABLO also resulted in a slight induction of survivin (Fig. 7A). Despite the up-regulation of survivin, Apo2L/TRAIL-mediated apoptosis of prostate tumor cells was still enhanced, suggesting that survivin is not a dominant resistance factor against Apo2L/TRAIL-mediated apoptosis in prostate tumor cells or that the level of induction was not sufficient to block the enhancement of killing.

Because the release of Smac/DIABLO from mitochondria can only be induced by treatment with Apo2L/TRAIL (Fig. 6), we examined whether the high level of Smac/DIABLO release in the Smac/DIABLO transfectant induced by Apo2L/TRAIL would greatly reduce the level of IAP family proteins. As seen in Fig. 7B, right panels, IAP family proteins (XIAP, c-IAP1, and c-IAP2) were reduced by Apo2L/TRAIL treatment in the Smac/DIABLO transfectants. Such changes were not observed in the untreated cells and vector transfectants. Other antiapoptotic proteins (FLIP, and Bcl-2) were not affected. In Act D-pretreated cells, Apo2L/TRAIL also reduced the level of c-IAP-2 slightly, whereas the levels of other antiapoptotic proteins were unchanged.

Discussion

In this study, we found that overexpression of Smac/DIABLO could render resistant tumor cells sensitive to Apo2L/TRAIL-
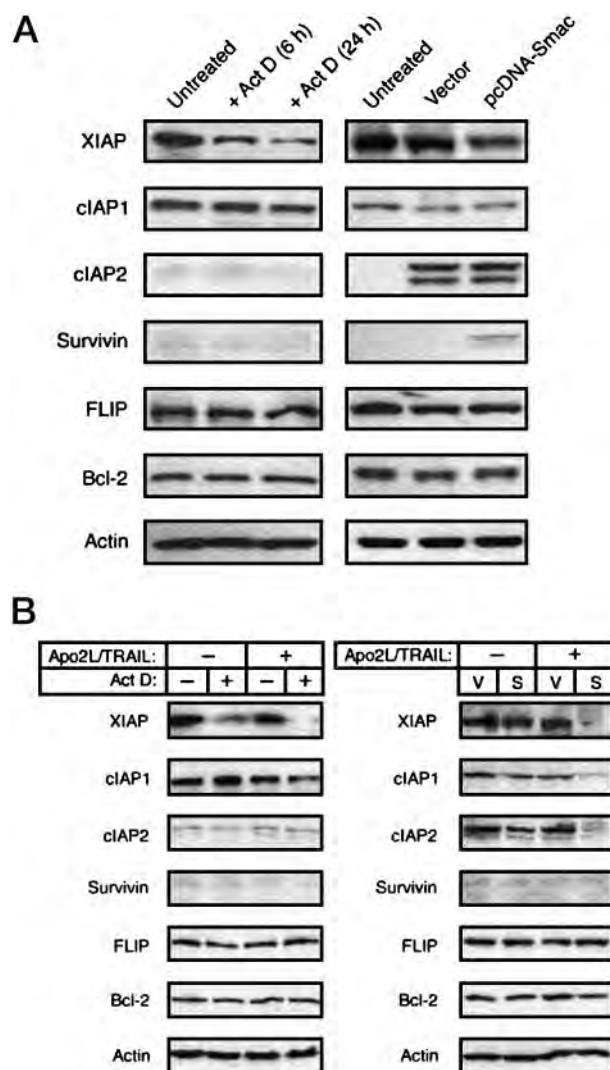
Fig. 7. Comparison of various antiapoptotic protein levels in Act D-treated cells and Smac/DIABLO transfectants. A, effects of Act D (100 ng/ml) and Smac/DIABLO overexpression on the protein levels of various antiapoptotic proteins. Protein lysates were prepared from cells treated with Act D (100 ng/ml) for 6 or 24 h and cells transfected with pcDNA3 (vector) and pcDNA3-FLAG-Smac/DIABLO for 24 h. The protein levels of IAP family members (XIAP, c-IAP1, c-IAP2, and survivin), FLIP, and Bcl-2 were examined by immunoblotting analysis. β-Actin was determined to ensure equal loading of protein lysates. B, effects of Apo2L/TRAIL (10 ng/ml) on the protein levels of various antiapoptotic proteins (XIAP, c-IAP1, c-IAP2, survivin, FLIP, and Bcl-2) in Act D-pretreated cells and Smac/DIABLO transfectants. +, cells pretreated with Act D (100 ng/ml) for 6 h before the addition of Apo2L/TRAIL (10 ng/ml). Lanes V and S indicate cells transfected with the vector pcDNA3 (Lanes V) or pcDNA-FLAG-Smac/DIABLO (Lanes S). Lysates were prepared after a 6-h treatment with Apo2L/TRAIL and then subjected to immunoblotting analysis.

mediated apoptosis. We compared the apoptotic signaling events in the Apo2L/TRAIL-mediated apoptosis pathway (release of cytochrome c and caspase activation) in both Act D-sensitized cells and Smac/DIABLO transfectants. Apo2L/TRAIL activated caspases-8, -9, and -3 in both Act D-sensitized cells and Smac/DIABLO transient transfectants, suggesting that activation of caspases was necessary to result in apoptosis. However, Apo2L/TRAIL induced the re-

lease of cytochrome *c* and Smac/DIABLO in both resistant control cells and sensitized cells. This result shows that the predominant resistance of prostate cancer cells is present downstream of the mitochondrial events, suggesting that IAP family proteins are the prime candidate proteins that confer prostate tumor immunoresistance. By comparing the protein expression patterns induced by Apo2L/TRAIL in Act D-sensitized cells and Smac/DIABLO transient transfectants, we found that down-regulation of XIAP was the common feature found in both cell systems. Thus, XIAP could be a potential target for overcoming Apo2L/TRAIL resistance, and up-regulation of Smac/DIABLO may serve as a potential effective modifying signal to immunosensitize resistant prostate tumor cells to apoptosis.

The identification of Smac/DIABLO as a neutralizing inhibitor of IAP family proteins has revealed additional complexities in the regulation of tumor sensitivity to apoptosis. Similar to cytochrome *c*, Smac/DIABLO is characterized as a mitochondrial protein, and it is released from the mitochondria when the cells received proper death signals (28, 29). Normally, Smac/DIABLO is produced as a precursor protein that contains a MTS and remains nonapoptotic. The proapoptotic function of Smac/DIABLO is attained when its MTS is cleaved after being transported to the mitochondria. Further analysis revealed that the 5-amino acid peptide AVPIA at the NH_2 terminus, which is exposed after cleavage of MTS, is thought to be responsible for the interaction with the baculovirus IAP repeat 3 domain of XIAP and inhibition of IAP function (36). Contrary to this finding, another report documented that the proapoptotic function of Smac/DIABLO does not depend on its IAP-neutralizing domain (37). In light of this study, whereas our results have suggested that Smac/DIABLO may exert its sensitizing effect by down-regulating XIAP, inhibition of other non-IAP resistance factors may be important for sensitization by Smac/DIABLO. Further detailed analysis of IAP-Smac/DIABLO interaction will determine whether indeed XIAP is one of the predominant resistance factors that were down-regulated by Smac/DIABLO.

The containment of Smac/DIABLO inside the mitochondria could be also important for the inhibition of apoptosis. It is unclear at this point whether there is a difference in the mechanisms for the release of cytochrome *c* and Smac/DIABLO. In CL-1 tumor cells, Apo2L/TRAIL was able to induce simultaneous release of cytochrome *c* and Smac/DIABLO, but the release of both molecules was still not sufficient to induce apoptosis. Therefore, the endogenous level of Smac/DIABLO in prostate tumor cells may not be adequate to neutralize the negative regulation on apoptosis. When the level of Smac/DIABLO was up-regulated in the transient transfectants, the sensitivity to Apo2L/TRAIL was restored.

The inability to induce apoptosis by translocation of cytochrome *c* and endogenous Smac/DIABLO induced by Apo2L/TRAIL highlights the importance of the inhibition of downstream apoptotic events in tumor resistance. In our studies, the induction of apoptosis and activation of effector caspases are possibly dictated by the level of IAPs. In CL-1 tumor cells, sensitization to Apo2L/TRAIL-mediated apoptosis is associated with down-regulation of IAPs, in particular, XIAP, by Act D or a high level of Smac/DIABLO. Other studies have also shown that overexpression of IAP family proteins is associated with poor responsiveness to apoptosis-inducing therapies. In leukemia patients, a high level of XIAP in leukemia tumor cells correlates with poor survival rates (38). In the same study, the expression of XIAP and c-IAP-1 was found to be the most predominant in a panel of 60 human tumor cell lines (38). Furthermore, XIAP was also found to be highly expressed in resistant ovarian cancer cells to chemotherapy and radiation, and treatment of ovarian cancer cells with antisense XIAP reversed the tumor resistance (39, 40). In addition to the downstream inhibitors such as XIAP, upstream resistance factors such as Akt, which affects BID cleavage, are also critical for Apo2L/TRAIL resistance (41, 42). These findings, together with our study, underscore the importance of death signal amplification through the mitochondrial pathway in the killing of prostate tumor cells by Apo2L/TRAIL.

Tumor resistance to conventional therapies remains a major problem today. To solve this problem, we previously proposed an approach that involves two complementary signals to restore a functional apoptotic pathway that can be used by immunotherapy (27, 43). Similar to the approach in which we used Act D and Apo2L/TRAIL as our two complementary signals to kill resistant prostate tumor cells, the combination of Smac/DIABLO overexpression and Apo2L/TRAIL treatment is an equally effective option. This finding confirms that the success of this combinatory therapeutic approach requires the identification and direct reversal of the apoptotic signaling block. We predict that any method that counteracts the function of the identified resistance factor should serve as an effective complementary signal with Apo2L/TRAIL to cause a potent anti-prostate tumor killing. The immunosensitization by direct down-regulation of resistant proteins serves as a potential approach to enhance both chemotherapeutic and immunotherapeutic responses.

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Immunosensitization of resistant human tumor cells to cytotoxicity by tumor infiltrating lymphocytes

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Abstract. Most anti-cancer therapies induce apoptotic cell death, but a major barrier to long-term cancer treatments is the generation of apoptosis-resistant tumor cells. Tumor cells that become resistant to one therapy are usually cross-resistant to subsequent therapies, including those with different cellular/molecular targets, suggesting that resistant tumor cells acquire modifications of the general apoptotic pathway. Most solid tumors are characterized by infiltration of lymphocytes (tumor infiltrating lymphocytes, TIL), which may serve as a basis for new strategies to generate tumor specific lymphocytes. However, TIL frequently are unable to kill autologous tumor cells suggesting that they are anergic/tolerant. It is possible that the TIL are functional but the tumor cells are resistant to TIL-mediated apoptotic pathways. Previous findings revealed that resistant tumor cells can be sensitized with cytokines or subtoxic concentrations of chemotherapeutic drugs and restore killing by cytotoxic lymphocytes. In this study, we examined whether TIL can kill autologous and allogeneic tumor cells following sensitization with chemotherapeutic drugs. Renal and prostate cancer-derived TIL were cytotoxic to chemosensitized resistant tumor cells. Killing by TIL was found to be perforin-dependent and perforin-independent. These findings demonstrate that combination drug and immunotherapy may be able to overcome tumor cell resistance to killing by TIL. Further, *in vivo* sensitization of drug-resistant tumor cells by subtoxic doses of sensitizing chemotherapeutic drugs may result in tumor regression by the host immune system.

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Introduction

Most treatments designed to kill cancers cells (i.e., radiation, chemotherapy) induce apoptotic cell death, but tumors frequently relapse that are unresponsive to further therapy, and the prognosis for the patient is poor. Furthermore, relapsed tumor cells are usually cross-resistant to other, unrelated therapeutic modalities, suggesting a general defect in cellular apoptotic pathways. Thus, we hypothesize that new anti-cancer therapies, such as gene and immunotherapy, which frequently target or utilize apoptosis-mediated lytic pathways, must first overcome tumor cell resistance to killing or they will also ultimately prove to be ineffective (1-5). One important consideration of immunotherapy has been the difficulty in generating a sufficient number of tumor specific lymphocytes; these cells frequently require exogenous stimulation (e.g., IL-2 activation), suggesting that the immune system is anergic or tolerant. However, it is equally likely that the tumor cells have become resistant to killing by the immune system, thereby explaining the inactivity of the immune response.

The identification of tumor specific lymphocytes has resulted in new strategies based on mounting a sustained and effective anti-tumor immune response (6-9). These strategies assume that a suitable number of specific anti-cancer effector cells can be isolated and stimulated against specific antigens and that the targeted tumor cells are sensitive to lymphocyte-mediated killing. However, most immunotherapy strategies are limited to appropriate MHC/antigen expressing tumor cells, suitable number of precursor lymphocytes, and by the toxic side affects of treatment (e.g., IL-2 cytokine therapy). Confounding this, tumor cells that down regulate MHC or antigen expression or become resistant to lymphocyte-mediated cytolytic pathways will avoid being killed.

Lymphokine-activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) are promising immuno-therapeutic strategies to treat cancers (10-12). LAK and TIL have been shown to kill autologous and allogenic tumor cells through the perforin/granzyme degranulation pathway and the TNF-family of apoptosis inducing ligands (FasL, TNF-α, TRAIL) (1,13-15). However, most strategies utilizing TIL and LAK have focused on mechanisms to proliferate and activate cytotoxic lymphocytes capable of recognizing and killing tumor cells, but relatively few studies address the underlying sensitivity to effector cells (16). In this study, killing of drug sensitized cell lines via Ca^{++}-independent (Fas/FasL)

and dependent (perforin/granzyme) cytolytic pathways by TIL from hormone refractory prostate cancer was examined. In addition, the cytotoxicity of renal-derived TIL against sensitized autologous tumor cells was also assayed. We propose an alternate strategy in which autologous tumor cells are sensitized by subtoxic concentrations of chemotherapeutic drugs (i.e., CDDP, VP-16) to killing mediated by cytotoxic lymphocytes. Furthermore, we hypothesize that this strategy will be able to induce tumor cell killing independent of specific immunotherapy strategies, such as cytokine activation.

Materials and methods

Cell lines. The human hormone-independent prostatic carcinoma cell lines, DU145 and PC-3, the hormone-dependent prostatic carcinoma cell line, LNCaP, and the renal carcinoma line, R11, were obtained from Dr Arie Belldegrun (Department of Urology, Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA). The AD10 cell line is an adriamycin (ADR)-resistant, multidrug resistant (MDR) pheno-type-expressing subline of the ovarian carcinoma cell line, A2780, and was obtained from Dr Ozols (Fox Chase Cancer Center, Philadelphia, PA). All cells were cultured in 10% heat-inactivated fetal calf serum (Atlanta Biologicals, Norcross, CA) added to RPMI-1640 (Gibco, Grand Island, NY) with 1% Na-pyruvate (Gibco), 1% non-essential amino acids (Gibco) and 1% Fungi-bact solution (Irvine Scientific, Santa Ana, CA), which contains 10000 U/ml penicillin G, 10 µg/ml streptomycin and 25 µg/ml fungizone and grown in a humidified atmosphere at 37˚C and 5% CO_2.

IL-2 (Hoffman La Roche, Nutley, NJ)-activated LAK cells were prepared from peripheral blood of a renal cancer patient (patient 1). Briefly, whole blood was collected into sterile tubes with 0.5 ml sterile heparin. PBMC were isolated by density centrifugation over a Ficoll-Hypaque density gradient (LSM, Durham, NC) and washed 3 times in sterile PBS. Cells were incubated in the presence of 1000 U/ml rhIL-2 (Hoffman La Roche) for at least 7 days prior to use. Human TIL cells were supplied by Dr A. Belldegrun from renal and prostate carcinomas and prepared as previously described (17). Briefly, fresh tumors were obtained from the operating room, minced, and digested overnight in RPMI-1640 (Cellgro, Washington, DC) with 0.01% hyaluronidase type V, 0.002% DNase type II, 0.1% collagenase type IV (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine (Gibco, Grand Island, NY), and 50 µg/ml gentamycin. Single cell suspensions (a mixture of tumor and TIL) were centrifuged over single step Ficoll-Hypaque density gradient (LSM). Tumor cells and TIL were retrieved from the gradient interface, washed, counted, and cultured in 75 ml tissue culture flasks (Costar, Cambridge, MA) at a density of 0.5x10⁶ cells/ml in RPMI-1640 medium + 10% heat-inactivated human AB serum (Irvine Scientific, Santa Ana, CA), 50 IU/ml penicillin, 50 µg/ml streptomycin (JHR Biosciences, Lenexa, KS), and 2 mM L-glutamine (1,3). Prostate-derived TIL were activated with 400 U/ml rhIL-2 for at least 14 days prior to use in cytotoxicity assays.

Renal TIL and autologous tumor cell fractions (patients 1 and 2) were isolated from single cell suspension of fresh tumors from two patients as described by Mizutani *et al* (16). Briefly, tumors were digested as described above and single

cell suspensions were layered on a discontinuous gradient consisting of 5 ml of 100%, 10 ml of 75% and 10 ml of 50% Ficoll-Hypaque density gradient (LSM) in 50 ml tubes and centrifuged at 400 g for 25 min. The lymphocyte-rich mono-nuclear cells were collected from the 100%-75% interface and autologous tumor cells from the 75%-50% interface (16). The purity of each fraction was determined by microscopy and was >90%. The tumor cells were then washed and cultured in 75 ml flasks at a density of 0.5x10⁶ cells/ml in RPMI-1640 medium + 10% heat-inactivated human AB serum (Irvine Scientific, Santa Ana, CA), 50 IU/ml penicillin, 50 µg/ml streptomycin (JHR Biosciences, Lenexa, KS), and 2 mM L-glutamine. The TIL fraction was incubated as above in the absence of rhIL-2 or with either low-concentration rhIL-2 (10 U/ml) or high-concentration rhIL-2 (1000 U/ml) for at least 7 days prior to use.

Reagents. Cisplatin (CDDP), EGTA, ionomycin, $MgCl_2$ and PMA were purchased from Sigma. $Na_2{}^{51}CrO_4$ was purchased from Amersham (Arlington Heights, IL). Stock solutions of CDDP were routinely prepared in dimethyl sulfoxide (DMSO), while PMA and ionomycin were prepared in ethanol.

Cell-mediated cytotoxicity assay. TIL and LAK cells were washed 3 times in PBS and resuspended at a final concentration of 10⁶ cells/ml and used immediately in the cytotoxicity assay. Tumor cells were trypsinized for 5 min, collected and washed once in PBS. Target tumor cells were incubated in 100 µCi of $Na_2{}^{51}CrO_4$ for 1 h at 37˚C and 5% CO_2, washed 3 times in medium, and 10⁴ cells were added to flat-bottom 96-well culture plates in the presence or absence of chemotherapeutic drugs (0.1-10 µg/ml). At the time of the experiment, the drug-containing medium was removed and 100 µl of fresh medium + 10% FCS was added to each well. Effector TIL or LAK cells (100 µl) in the presence or absence of 3 mM EGTA/2 mM $MgCl_2$ (pH 7.0) were added at the indicated E:T ratio. Plates were centrifuged and incubated for 5-7 h at 37˚C and 5% CO_2. Following incubation, 100 µl of supernatant was harvested from each well and counted in a Beckman gamma-4000 gamma counter. Total ⁵¹Cr-release was determined by lysing target cells with 50 µl of 10% SDS buffer and collecting 150 µl for count. The percent specific ⁵¹Cr-release was determined as follows:

$$\% \ {}^{51}\text{Cr-release} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

Flow cytometry. Cells were incubated in the presence or absence of the chemotherapeutic drug, CDDP (0.1-10 µg/ml), for 18 h at 37˚C and 5% CO_2. Following incubation, the cells were trypsinized for 5 min, collected and washed 3X in PBS + 10% FBS. For staining, 100 µl of 1-2x10⁶ cells/ml were added to 96-well U-bottom plates (Costar). Fas expression was assayed by 10 µg/ml of PE conjugated mouse anti-human Fas monoclonal antibody (IgG subclass, clone ZB4) (Pharmingen), or 10 µg/ml of normal mouse IgG (Pharmingen) added to the cells for 30 min on ice. Cells stained with non-conjugated primary antibody were washed 3X in PBS then resuspended in 50 µl 1X PBS containing goat anti-mouse IgG FITC-

Table I. Fas expression and Fas antibody-mediated killing (clone CH11, IgM) of human tumor cell lines.

Cell line	Source	Fas expression	Fas antibody-mediated killing	Fas antibody-mediated killing following drug treatment
AD10	Ovarian	+	-	+
DU145	Prostate	+	-	+
LNCaP	Prostate	+	-	-
PC-3	Prostate	+	-	+
R11	Renal	+	-	+
-	Fresh renal tumor	+	ND	ND
-	Fresh renal tumor	+	ND	ND

Fas expression was assayed by flow cytometry and CH11 sensitivity was determined by 18-h ^{51}Cr-release assay. For sensitization study, target cells were treated for 18 h with subtoxic concentrations of CDDP. Results have been previously reported (1,18). +, indicates positive effect (Fas expression, CH11 antibody-mediated killing). -, indicates negative effect (no Fas expression, no CH11 killing). ND, indicates sensitivity not determined.

Figure 1. Renal-derived TIL kill prostate tumor cell lines via both Ca^{++}-independent and dependent-cytolytic pathways. Cytotoxicity was determined by the ^{51}Cr-release assay at a 10:1 E:T ratio in the absence or presence of EGTA/MgCl$_2$. Target cells, DU145, PC-3 or LnCAP were pretreated for 18 h with 10 μg/ml CDDP prior to assay. *P<0.05 of control treated target cells versus target cells treated with CDDP.

conjugated antibody (Immunotech, Miami, FL) for 30 min on ice. Following incubation, the cells were washed 3X with PBS and assayed on an Epics-XL MCL flow cytometer (Coulter, Miami, FL).

Statistical analysis. All experiments were conducted on at least 3 separate occasions unless otherwise noted. All values are presented as the mean ± standard deviation of triplicate samples. Analysis of variance (one-way or two-way ANOVA) was used to test for significance. Pairwise analysis was performed by the Bonferroni/Dunn post hoc tests. P<0.05 was determined to be significantly different from the control.

Results

Previous studies in our laboratory have shown that CDDP sensitizes the ovarian cell line, AD10, and the prostate carcinoma cell lines, DU145 and PC-3, but not LNCaP, to anti-Fas antibody-mediated killing (1,2,18,19). Therefore, we examined the expression of Fas on target cells as a first step in order to characterize the sensitivity of tumor cell lines to Fas-mediated cytotoxicity by TIL. The results summarized in Table I demonstrate that most tumors studied express Fas receptor on the cell surface.

Cytotoxic T-lymphocytes (CTL), LAK and TIL cells utilize the perforin/granzyme and Fas/FasL apoptotic pathways to kill tumor cell lines (1,21). However, it is not clear what signals activate these individual pathways, if they act independent of each other, or if they are preferentially triggered by autologous tumor cells. Therefore, we assayed the pathways used by TIL
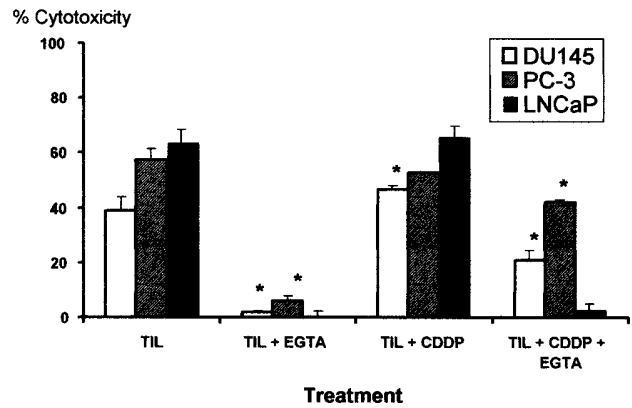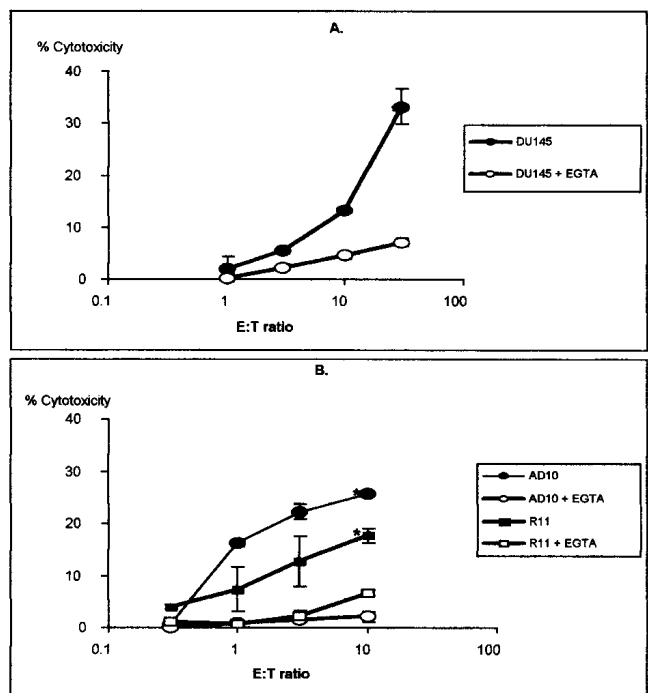

Figure 2. Prostate-derived TIL from a hormone refractory patient (patient 2) kills target cells via the perforin-mediated pathway. Cytotoxicity was determined by the ^{51}Cr-release assay at indicated E:T ratios in the absence or presence of EGTA/MgCl$_2$. (A), DU145, prostatic carcinoma cell line. (B), AD10, ovarian carcinoma, and R11, renal carcinoma cell lines. *P<0.05 compared to EGTA treated cells.

isolated from patients with prostate tumors to kill tumor cell lines. IL-2-activated prostate-derived TIL effectively killed all the tumor cell lines (DU145, PC-3, and LNCaP) tested in a non-MHC restricted manner (Fig. 1). However, treatment with the Ca^{++}-chelator, EGTA/MgCl$_2$, which blocks the Ca^{++}-dependent perforin-mediated pathway, but not the Ca^{++}-independent Fas/FasL pathway (21) blocked TIL-mediated
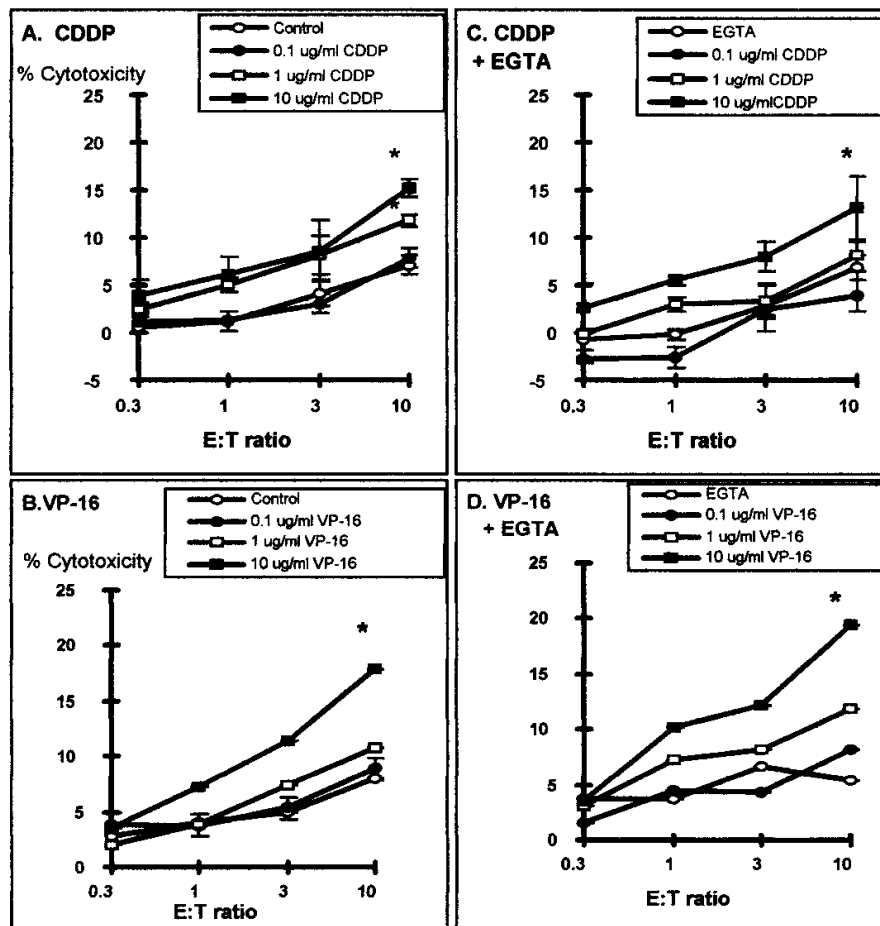
Figure 3. CDDP sensitizes DU145 cells to killing by prostate derived TIL from hormone refractory patient (patient 1). Cytotoxicity was determined by the ^{51}Cr-release assay at indicated E:T ratios in the absence (A and C) or presence (B and D) of EGTA/MgCl$_2$. Target cells were treated for 18 h with indicated concentration of CDDP (A and B) or VP-16 (C and D) prior to assay. *P<0.05 compared to control (no drug) cells.

killing of target cells (Fig. 1). However, overnight sensitization of DU145 and PC-3 with CDDP resulted in TIL-mediated killing by a Ca^{++}-independent mechanism (Fig. 1). TIL from a second patient was able to kill DU145 cells and cytotoxicity was blocked by EGTA, suggesting perforin involvement (Fig. 2A). In addition, TIL from this patient also killed AD10 and R11 cells (P<0.05) (Fig. 2B).

In contrast, IL-2-activated TIL-derived from another patient exhibited little or no killing of DU145 target cells in the presence or absence of EGTA (Fig. 3). These finding suggest that these TIL were unable to trigger the perforin/granzyme cytotoxic pathway. However, subtoxic concentrations of CDDP or VP-16 resulted in significant sensitization of DU145 cells to these TIL in a Ca^{++}-independent manner (P<0.05) (Fig. 3).

Our previous findings demonstrate that LAK and TIL can kill sensitized tumor cells by the Fas/FasL apoptotic pathway (1,3). In addition, it has been shown that freshly isolated cytotoxic lymphocytes express FasL (22,23), and some chemotherapeutic drugs have been shown to upregulate FasL expression on both cytotoxic lymphocytes and tumor cells (24,25). Therefore, we tested if CDDP could sensitize tumor cells to TIL-mediated killing in the absence of IL-2 activation. To confirm whether CDDP can sensitize autologous tumor cells to killing by non-IL-2 treated TIL, we separated TIL
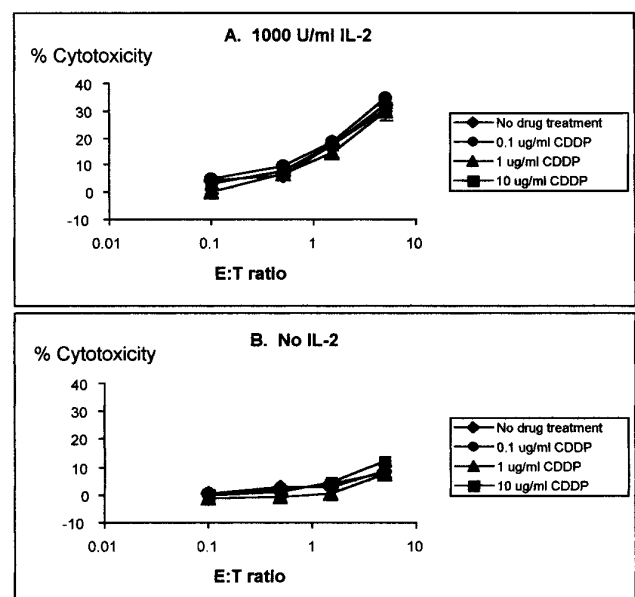

Figure 4. Renal-derived TIL from patient 1 were activated with IL-2 (1000 U/ml) and kill fresh autologous tumor cells (patient 1). (A), TIL-activated with 1000 U/ml IL-2. (B), Non-IL-2-activated TIL. Cytotoxicity was determined by ^{51}Cr-release assay at indicated E:T ratio. Target cells were treated for 18 h with indicated concentration of CDDP prior to assay.
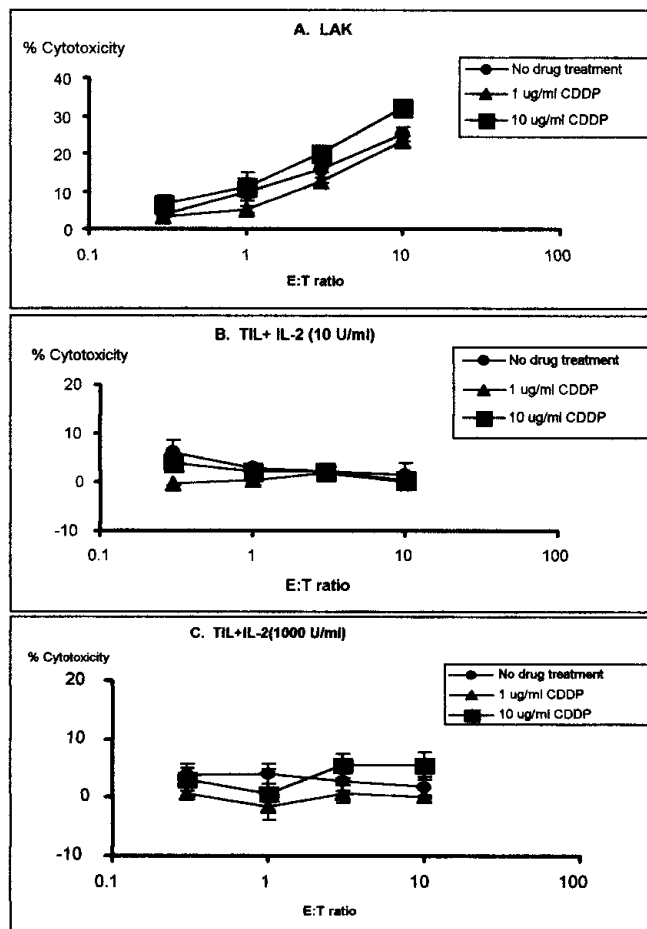
Figure 5. Renal-derived TIL from patient 2 do not kill fresh autologous tumor cells. (A), Patient-derived LAK-activated with 1000 U/ml IL-2. (B), TIL-activated with 10 U/ml IL-2. (C), TIL-activated with 1000 U/ml IL-2. Cytotoxicity was determined by ^{51}Cr-release assay at indicated E:T ratios. Target cells were treated for 18 h with indicated concentration of CDDP prior to assay.

and tumor cells from two patients with renal carcinoma and assayed for cytotoxicity. Autologous tumor cells derived from patient 1 were killed by recombinant human IL-2 (1000 U/ml) activated TIL, but not by non-IL-2 treated TIL (Fig. 4). In contrast, TIL-derived from patient 2 were not able to kill autologous tumor cells, although patient-derived LAK were cytotoxic (Fig. 5).

Discussion

One of the major problems facing the treatment of cancer is the development of resistant tumor cells. This has led to the exploration of new and more effective treatments for curing cancer, such as gene and immunotherapy. However, it is reasonable to assume that these approaches may generate resistant tumor cells and that not all tumor cells will be sensitive to killing by these new treatments. Therefore, we hypothesize that strategies designed to overcome tumor cell resistance to killing will enhance the overall effectiveness of anti-cancer therapies. TIL have been shown to be non-cytotoxic and do not exert anti-tumor activity. The general

belief is that the TIL might have acquired an anergic phenotype and thus, are incapable of mediating cytotoxicity (26). However, another conceivable alternative is that TIL are functional, but that the tumor cells are resistant to cytotoxicity. The data presented here provide supportive evidence to our previously reported findings that TIL are capable of killing tumor cells via both the perforin/granzyme and dependent and independent pathways (1,3). However, in some cases, TIL are unable to exert detectable cytotoxicity against target cells (Fig. 3 and 5). In those cases, it may be possible to sensitize tumor cells to killing by the perforin-independent apoptotic pathway using subtoxic concentrations of chemotherapeutic drugs. In addition, our findings suggest that sensitizing drug therapy may potentiate TIL-mediated killing of resistant tumor cells (Fig. 1).

Most immunotherapy strategies are designed to elicit a specific anti-tumor immune response from cytotoxic lymphocytes (6,10,27). Some of these approaches have shown promising results for treating cancers and some have begun clinical trials (28-30). However, in general, these treatments have a response rate of less than 50%. The relatively low frequency of success, due to such factors as low precursor frequencies of tumor specific lymphocytes, tumor-mediated immunosuppression, down regulation of MHC/antigen expression, and killing of activated effector cells via FasL expression on tumor cells all may explain failure of these therapies (reviewed in refs. 4,31). We hypothesize that one major source of tumor evasion is due to resistance to cytotoxic lymphocyte-mediated killing mechanisms (1,31).

The ultimate measure of any anti-cancer therapy is the ability to kill tumor cells. It may be reasonable to assume that some tumor cells are resistant to some/all cytotoxic-lymphocyte-mediated pathways and/or preferentially trigger only those pathways for which they are resistant. In this study we present data suggesting that the primary mode of TIL-mediated killing is via the perforin/granzyme cytotoxic pathway, as would be expected since most of the target cell lines we tested are resistant to anti-Fas antibody-mediated killing (Table I) (1,2). However, we were able to sensitize some tumor cells to a non-perforin-mediated component of cytotoxic lymphocytes by treatment with subtoxic concentrations of chemotherapeutic drugs (Figs. 1 and 3).

One clinical manifestation of drug-mediated sensitization is the potential ability to enhance TIL- and LAK-mediated killing of resistant tumor cells. Furthermore, one limiting factor in TIL- and LAK-mediated immunotherapy is the isolation, purification and activation of a sufficient number of effector cells to mediate an immune response. For example, prostate-derived TIL from one patient, which were unable to kill untreated DU145 cells, could kill drug sensitized DU145 cells by the Ca^{++}-independent FasL-mediated pathway (Fig. 3). This suggests that drug-mediated sensitization of tumor cells could potentiate the effectiveness of immunotherapy through the sensitization of certain tumor cells to FasL-mediated killing. In addition, freshly isolated TIL and NK cells, which express FasL, may also kill sensitized tumor cells, in situ, without any further activation treatments (i.e., IL-2 activation) (16,32).

Our data did not demonstrate that fresh renal tumor cells could be sensitized to non-perforin-mediated killing by TIL (Fig. 4), most likely by FasL, despite the fact that all tumor

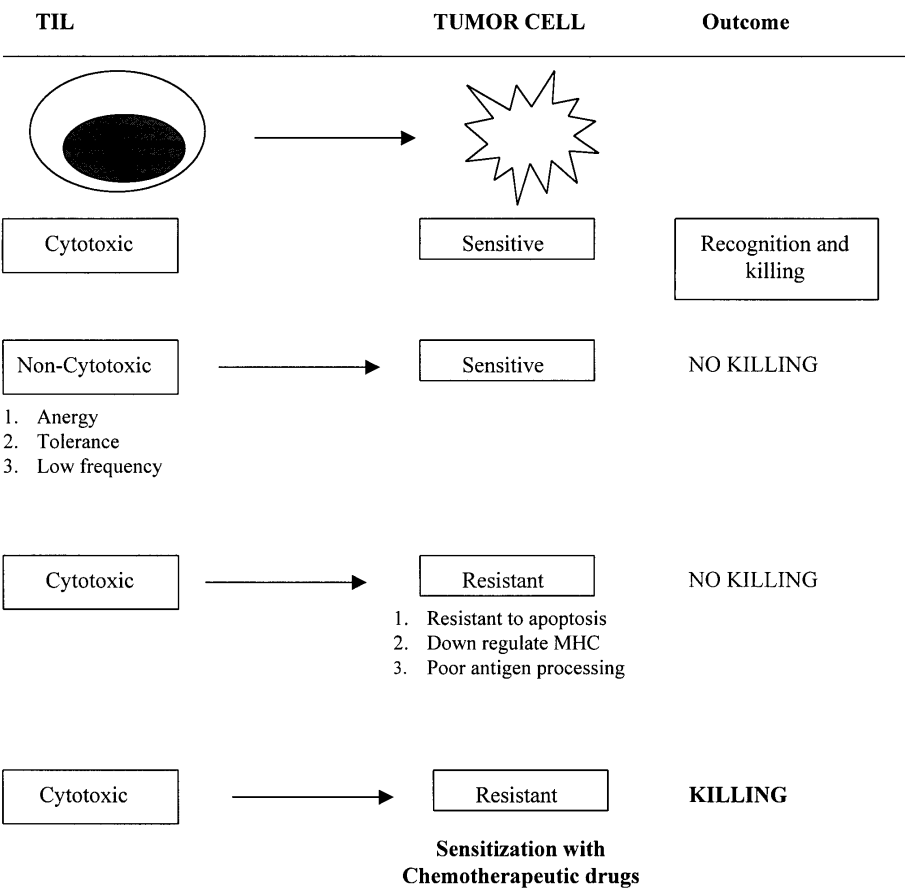| TIL	TUMOR CELL	Outcome

Figure 6. Schematic diagram of several possible mechanisms by which tumor cells can avoid surveillance and killing mediated by cytotoxic lymphocytes. Tumor cell/TIL interactions result in a number of potential outcomes, including: 1) killing of tumor cell, 2) inactivation of TIL, 3) resistance to tumor cell to TIL-mediated cytotoxic pathways. TIL that have become anergic or tolerant typically require activation or stimulation (e.g., IL-2) to kill tumor cells. Tumor cells that either down regulate MHC/antigen complex or become resistant to lymphocyte-mediated apoptotic pathways (e.g., Fas/FasL, perforin/granzymes) will not be killed by TIL, even following activation. However, sensitization of tumor cells may result in TIL-mediated killing. Finally, low precursor levels of anti-tumor TIL will limit killing. However, sensitization of tumor cells to non-MHC-mediated cytotoxic pathways will result in the potential for non-tumor specific TIL to kill sensitized tumor cells.

cells tested expressed cell surface Fas (Table I). Further, we were unable to show that tumor cells could be sensitized to non-IL-2 TIL in bulk culture (Figs. 4 and 5). These findings do not invalidate our essential premise, since it is possible that other factors, such as immunosuppressive cytokines, are also involved. In addition, other chemotherapeutic drugs and/ or cytokines may be more effective than CDDP in sensitizing autologous tumor cells to immunotherapy. Finally, our study size was very small, and thus the findings may not be representative of the majority of patients. However, for patients with drug and immune resistant tumor cells, sensitization therapy may overcome resistance to TIL-mediated apoptosis.

Clearly, drug-mediated sensitization may potentiate immunotherapy for some patients. We demonstrate that at least for some patients, TIL are unable to mount a cytotoxic response. However, subtoxic concentrations of CDDP or VP-16, can potentially sensitize these immunoresistant tumor cells to the TIL-mediated apoptotic pathways (Fig. 3). It should be noted, however, that not all tumor cell lines were susceptible to sensitization therapy. These studies are the first to examine the potential ability of sensitization to modify patient derived TIL-mediated killing of autologous tumor cells,

and support our hypothesis that drug-mediated sensitization can enhance immunotherapy.

Our past and present findings indicate that TIL may be endowed with cytotoxic activity and that the adjacent tumor cells have acquired resistance to cytotoxicity. However, if tumor cells resistance is overcome, such tumor cells revert to being sensitive and are killed by the TIL (Fig. 6). These findings suggest that one may use clinically sensitizing cytotoxic drugs, either intratumorally or systemically, to sensitize resistant tumor cells. Further, the sensitization activates both MHC-restricted (perforin/granzyme) and non-MHC-restricted (Fas/FasL) cytotoxic mechanisms of killing by cytotoxic lymphocytes (TIL, LAK, CTL).

Acknowledgements

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QSAR of Apoptosis Induction in Various Cancer Cells

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Abstract—In continuing our QSAR study of apoptosis, we consider in this report the action of phenolic compounds on Ramos cells (non-Hodgkins B-cell lymphoma): the effect of O-8-thapsigargin analogues on human prostate cancer cells, Tsu-Pr-1 and the induction of apoptosis of a complex set of congeners on human fibrosarcoma cells HT 1080. The human prostate cancer cells activity is very similar to that of the Ramos cells. While the QSAR for the fibrosarcoma cells resembles that of our earlier study with L1210 leukemia cells. The two different types of QSAR suggest at least two quite different types of receptors for the induction of apoptosis.
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Introduction

Programmed or physiological cell death, also known as apoptosis, is a unique type of cell death characterized by cytoskeletal disruption, cellular shrinkage, membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation. Being a genetically controlled process, apoptosis is susceptible to mutations, and dysregulation of the apoptotic machinery is frequently observed in numerous types of cancers.

Because of the close correlation between tumorigenesis and dysregulation of apoptosis, any therapeutic strategy aimed at specifically triggering apoptosis in tumor cells might have potential therapeutic applications. It is well established that exposure of cells to chemical toxicants, chemotherapeutic drugs or radiation perturbs cellular homeostasis including the induction of nucleotide excision and DNA double strand breaks. These events disturb normal cell cycle checkpoints and trigger the apoptotic machinery.

The emergence of resistance to conventional therapeutic strategies has encouraged the design and/or exploitation of novel chemicals with anti-cancer properties. The objective of the present study was to obtain quantitative structure–activity relationships (QSAR) using an array of phenol compounds to analyze their ability to induce apoptosis in L1210 murine lymphoma cell line.

In our studies to help elucidate the various mechanisms of action of phenols on living systems, or their parts,[1−3] it was decided to test a set of phenols on L1210 leukemia cells. From this study, QSAR 1 was formulated.[2]

$$\log 1/C = -0.19\ (\pm0.02)\ \mathrm{BDE} + 0.21\ (\pm0.03)\ \log P$$
$$+ 3.11(\pm0.10) \tag{1}$$
$$n = 52, \quad r^2 = 0.920, \quad s = 0.202, \quad q^2 = 0.909$$

BDE is the calculated homolytic bond dissociation energy for the OH bond. Using the Hammett parameter σ^+ gave a somewhat less satisfactory result: $r^2 = 0.895$, $n = 51$. This was due, at least in part, to some unusual phenols such as estradiol, estriol, diethylstilbestrol, etc. for which σ^+ values are not known and had to be estimated.

Actually this study was inspired by a publication from the EPA on the deformation of rat embryos in vitro by phenols.[3] We found the data to be correlated by σ^+. We decided that the rapidly growing embryo cells were producing large amounts of ROS (reactive oxygen species) and that these were converting the phenols to radicals that caused the initial damage. It was well known from studies in physical organic chemistry that radical formation from substituted phenols is correlated by σ^+. Hence we decided to study the action of phenols on fast growing cancer cells.

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With this background, it was decided to study some of the phenols on which QSAR 1 was based for their ability to cause apoptosis.[4]

$$\log 1/C = -3.61 \ (\pm 1.0) \ \text{Clog P} + 2.77 \ (\pm 0.65) \ \text{CMR}$$

$$-3.76 \ (\pm 1.3) \tag{2}$$

$$n = 11, \quad r^2 = 0.939, \quad s = 0.630, \quad q^2 = 0.892$$

outlier : Bisphenol A

C is the molar concentration causing 25% apoptosis in 24 h. In QSAR 1, C is the concentration causing 50% decrease in cell growth in 48 h. We were astonished that no electronic term could be found for QSAR 2. Obviously we have *much* to learn about how chemicals affect living systems. It is interesting that a period of 24 h seems to be enough to separate the two processes.

One interesting fact about QSAR 2 is that the most potent inducer of apoptosis was the normal female hormone estradiol. One wonders if one of its functions in women might be that of inhibiting the growth of abnormal cells such as cancer. After menopause the production of estradiol decreases and the incidence of cancer increases. Women are often given a supplement of female hormones to offset the decline.

Methodology

The experimental technique for QSAR 3 has been previously discussed.[4] The regression analysis including autoloading of all parameters was accomplished with the C-QSAR program.[5]

Results

As far as we can ascertain, QSAR 2 is the first published example for apoptosis, despite the fact that there is enormous interest in the subject, one of which is the search for anticancer drugs. Eq 2 supports this possibility. After the study on L1210 leukemia cells it was decided to study other types of cells. The first effort was made with 2F cells with the same phenolic compounds, however, no satisfactory QSAR could be

obtained. We now report results obtained using Ramos cells (Table 1) from which QSAR 3 was developed.

$$\log 1/C = 0.67 \ (\pm 0.21) \ \text{Clog P} + 0.37 \ (\pm 0.63)$$

$$n = 8, \quad r^2 = 0.910, \quad s = 0.201, \quad q^2 = 0.863$$

$$\tag{3}$$

outliers : 4-OCOCH$_3$-Phenol; 4-SMe Phenol;

diethylstilbesterol

There is a startlingly different result from QSAR 2 and indicates that interaction with a hydrophobic receptor is involved. However, there is no explanation for three of the four outliers. It is not surprising that diethylstilbesterol is an outlier, what was surprising is that it is well fit by QSAR 1 and 2. Of the well fit compounds, estradiol is also potent as in the case of QSAR 2.

In searching the literature for other data suitable for QSAR construction, that of Jakobsen et al.[6] attracted our attention. They reported the concentrations of 0–8-Thapsigargin analogues causing 50% loss of clonogenic survival of human prostate cancer TSU-PR-1 cells, that is, the ability to induce apoptosis, from which we formulated QSAR 4 (Table 2).

Table 1. Data for QSAR 3

	Compd	Log 1/C	Pred log 1/C	Dev	Clog P
1	Estradiol	2.79	2.88	−0.09	3.78
2	4-MeO-phenol	1.27	1.41	−0.14	1.57
3	4-C$_6$H$_5$O-phenol	2.67	2.74	−0.07	3.57
4	4-CH$_3$COO-phenol[a]	3.01	1.33	1.68	1.46
5	Bisphenol A	2.84	2.81	0.03	3.67
6	4-(Me)$_3$C-phenol	2.65	2.56	0.09	3.30
7	4-CN-phenol	1.44	1.43	0.01	1.60
8	Diethylstilbestrol[a]	2.89	3.66	−0.77	4.96
9	4-I-phenol	2.08	2.29	−0.21	2.90
10	Phenol[a]	3.10	1.35	1.75	1.48
11	4-MeS-phenol[a]	2.60	1.72	0.88	2.03
12	4-C$_3$H$_7$O-phenol	2.51	2.12	0.39	2.63

[a]Data points not used in deriving QSAR 3.

Table 2. Data for QSAR 4

|	Substituent	Log 1/C	Pred log 1/C	Dev	Clog P′
1	C$_3$H$_7$[a]	7.52	5.26	2.27	2.66
2	(CH$_2$)$_2$C$_6$H$_4$-4-NHCOCH(NH$_2$)CH$_2$CHMe$_2$	6.06	5.95	0.10	3.57
3	(CH$_2$)$_6$NH$_2$	4.96	5.03	−0.07	2.36
4	(CH$_2$)$_7$NH$_2$	5.42	5.43	−0.17	2.89
5	(CH$_2$)$_{10}$NH$_2$	6.13	6.64	−0.52	4.47
6	(CH$_2$)$_{11}$NH$_2$[a]	5.94	7.04	−1.11	5.00
7	(CH$_2$)$_{11}$NHCOCH(NH$_2$)CHMe$_2$	7.52	7.27	0.25	5.30
8	(CH$_2$)$_{11}$NHCOCH(NH$_2$)Me	6.55	6.56	−0.01	4.37
9	(CH$_2$)$_{11}$NHCOCH(NH$_2$)CH$_2$OH	6.05	5.79	0.26	3.36
10	(CH$_2$)$_{11}$NHCOCH(NH$_2$)CH$_2$C$_6$H$_5$[a]	6.68	7.65	−0.97	5.79

[a]Not used in the derivation of QSAR 4.
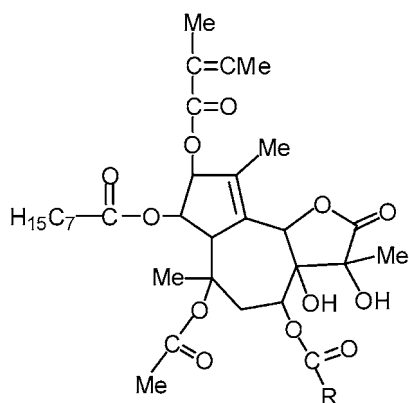
$$\log 1/C = 0.76\,(\pm0.30)\ \text{Clog}\ P' + 3.23\,(\pm1.15)$$

$$n = 7, \quad r^2 = 0.897, \quad s = 0.287, \quad q^2 = 0.786$$

$$outliers:\ R = C_3H_7; \tag{4}$$

$$(CH_2)_{11}NH_2;\ NHCO(CH_2)_{11}COCHCH_2C_6H_5$$
$$\underset{NH_2}{|}$$
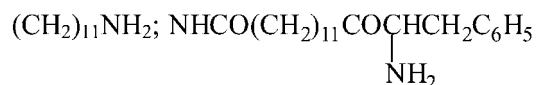
The Clog P values are very large, ranging from 5.33 to 8.8 while the statistics are good, three data points could not be included. All of the R except C_3H_7 contained an amino function that would be protonated under experimental conditions. For this reason the C_3H_7 cannot be expected to fit. P′ indicates that we have corrected the calculated log P by subtracting 3.5 from the calculated value. The figure 3.5 is the difference between Clog P and log P of RNH_2 measured at pH 7.4. This correction results in a more reasonable value for the intercept. It is the log P terms in equations 3 and 4 that are essentially identical, indicating similar receptors in the two different types of cancer cells. It is generally assumed that it is the perturbation of DNA that induces apoptosis.[7–9]

Next from the data of Keenan et al.[10] we have developed QSAR 5 for induction of apoptosis of human fibrosarcoma cells HT1080 (Table 3).
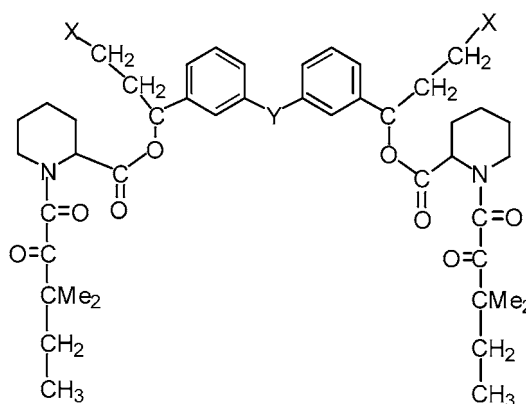
$$\log 1/C = -0.45\,(\pm0.17)\ \text{Clog}\,P + 0.35$$

$$\times\,(\pm0.13\,)\ CMR + 0.56\,(\pm3.9) \tag{5}$$

$$n = 12, \quad r^2 = 0.886, \quad s = 0.240, \quad q^2 = 0.717$$

outliers: see Table 3

C is the molar concentration of chemical inducing 50% apoptosis.

It is of interest that QSAR 5 is based on the same parameters with the same signs of the coefficients as QSAR 2 suggesting the same type of reaction center despite the grossly different chemical structures that are involved as well as the different type of cells. However, the size of the coefficients is much smaller.

From data from Christensen et al.[11] on the induction of apoptosis in human prostate cancer cells TSU-PR-1, QSAR 6 was formulated (Table 4).

Table 3. Data for QSAR 5

	Compd X	Y	Log 1/C	Pred log 1/C	Dev	Clog P	CMR
1[a]	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	8.22	7.41	0.81	9.95	32.33
2	4-OCH$_3$-C$_6$H$_4$	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	6.40	6.74	−0.34	10.47	31.10
3	3-C$_5$H$_4$N	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	7.52	7.44	0.09	7.64	29.44
4[a]	N-C$_4$H$_8$NO	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	6.00	7.78	−1.78	6.71	29.24
5	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	7.22	7.41	−0.19	9.95	32.33
6	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_3$NHCOCH$_2$	7.70	7.48	0.22	10.15	32.80
7	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_2$O(CH$_2$)$_2$NHCOCH$_2$	8.00	7.76	0.24	10.01	33.41
8	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_2$O(CH$_2$)$_2$O(CH$_2$)$_2$NHCO	7.82	8.11	−0.28	10.07	34.49
9	3,4-di-OCH$_3$-C$_6$H$_3$	(CH$_2$)$_2$NHCOCONH(CH$_2$)$_2$	7.10	7.10	0.00	10.64	32.33
10[a]	3,4-di-OCH$_3$-C$_6$H$_3$	(CH$_2$)$_2$NHCONH(CH$_2$)$_2$	8.22	6.66	1.56	11.22	31.83
11	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CH(OH)CH$_2$	6.15	6.44	−0.29	10.51	30.29
12	3,4-di-OCH$_3$-C$_6$H$_3$	(CH$_2$)$_2$O(CH$_2$)$_2$	6.46	6.20	0.25	11.40	30.75
13	3,4-di-OCH$_3$-C$_6$H$_3$	(CH$_2$)$_2$O(CH$_2$)$_2$O(CH$_2$)$_2$	6.70	6.55	0.15	11.46	31.83
14	3,4-di-OCH$_3$-C$_6$H$_3$	(CH$_2$)$_2$O(CH$_2$)$_2$O(CH$_2$)$_2$O(CH$_2$)$_2$	6.86	6.90	−0.05	11.52	32.91
15	3,4-di-OCH$_3$-C$_6$H$_3$	CH$_2$CONH(CH$_2$)$_2$NHCOCH$_2$	7.60	7.41	0.19	9.95	32.33

[a]Not used in the derivation of QSAR 5.

Table 4. for QSAR 6

	Substituent	log 1/C	Pred log 1/C	Dev	MgVol
1	COC_3H_7	7.52	7.51	0.01	4.98
2	H^a	5.00	8.26	−3.26	4.40
3	$CO(CH_2)_5CONH-(C_6H_3-3-NH_2-4-Me)$	5.51	5.70	−0.19	6.36
4	$CO(CH_2)_3CONH-(C_6H_3-3-NH_2-4-Me)^a$	4.78	6.07	−1.29	6.08
5	$CO-C_6H_4-4-NH_2{}^a$	5.40	7.14	−1.74	5.26
6	$CO(CH_2)_2-C_6H_4-4-NH_2$	6.55	6.77	−0.22	5.54
7	$COCH=CH-C_6H_4-4-NH_2$	6.96	6.83	0.13	5.50
8	$CO(CH_2)_3-C_6H_4-4-NH_2$	6.64	6.59	0.05	5.68
9	$CO(CH_2)_2-C_6H_4-4-NHCO_2C(Me)_3$	6.06	5.76	0.30	6.32
10	$COCH=CH-C_6H_4-4-NHCO_2C(Me)_3$	5.72	5.81	−0.09	6.28

^{a}Not used to derive QSAR 6.
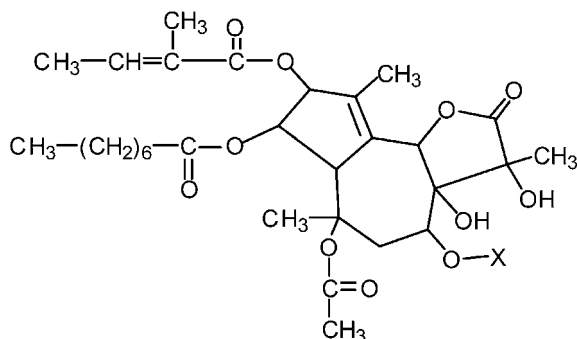
$$\log 1/C = -1.30\ (\pm 0.41)\ MgVol + 14.0\ (\pm 2.36)$$

$$n = 7, \quad r^2 = 0.932, \quad s = 0.203, \quad q^2 = 0.870$$

outliers : $X = H$; $CO(CH_2)_3CONH-C_6H_3-3$

$$-NH_2, 4\text{-Me}; \quad COC_6H_4-4-NH_2 \quad (6)$$

Again we find a very complex set of 'congeners' correlated by a simple QSAR with no sign of an electronic or hydrophobic interaction. MgVol is a parameter for the molecular volume of the molecule. The larger the substituent, the less effective it is in inducing apoptosis. Using CMR in place of MgVol yields a similar QSAR, with $r^2 = 0.914$.

Discussion

It is well established that chemotherapeutic drugs eradicate tumor cells via the induction of apoptosis, a genetic process of programmed cell death.[13] The effector cells of the immune system also utilize various apoptotic pathways (e.g., Fas ligand, TRAIL, and TNF-α) in killing their target cells.[13] Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with an intrinsic survival advantage and inherent resistance to apoptotic machinery. As a corollary to this, tumor cells become resistant to apoptosis induced by chemotherapeutic drugs as well as the immune system, which will result in their growth and expansion.[14] This has spurred the intriguing idea of exploitation of alternative chemical compounds capable

of the induction of apoptosis in otherwise resistant tumor cells. In the present study we have evaluated the apoptotic attributes of an array of phenol compounds on murine lymphoma cell line L1210. In addition, using quantitative structure function relationship (QSAR) equations, we have established a close correlation between the chemical structure of the compounds and their ability to induce apoptosis.

The most amazing aspect of QSAR 2 to 6 is that such heterogeneous 'congeners' can be correlated at all by simple QSAR. This was unexpected in the findings of QSAR 1 and 2, but it was even more surprising in QSAR 4 and 5. A perusal of the substituents in Tables 2, 3 and 4 finds that these are not at all simple. There is no way to employ Hammett parameters or steric parameters such as the sterimol group B1, B5 and L. Thus we are left with the general descriptors log P, CMR and MgVol.

The parameter CMR that is the calculated molecular refractivity is complex. It is based on the Lorentz–Lorenz equation:

$$MR = n^2 - 1/n^2 + 2\ (MW/d)$$

In this expression, n represents index of refraction, MW stands for molecular weight and d is density. Thus, n is a measure of the interaction of the electrons with light (polarizability) and MW/d is molar volume. Thus two properties are involved in CMR. Despite this complexity, we have found CMR^5 to be essential in the formulation of 1200 biological QSAR. We have recently discussed its properties in some detail.[15]

Over the years it is our experience in developing 9300 biological QSAR that one cannot take a newly formed equation very seriously. Only as one develops lateral validation by means of related biological QSAR or by establishing relationships with equations from physical organic chemistry can confidence be placed in a new QSAR. We have found such support in our current study. QSAR 3 and 4 both point to a hydrophobic receptor site, possibly DNA. Equations 2 and 5 contain the same terms with the same signs and thus point to a different hydrophilic site. Previously we noted[4] that a QSAR with −log P and +CMR terms was associated with mutagenic activity. No doubt DNA was involved.

Obviously none of the data sets considered so far are ideal for QSAR studies. That is structural changes are

so gross that there is little reason to assume that all members of a set are binding in the same mode to the same site. However, we now see that apoptosis can be treated via relatively simple equations. We hope that our results will encourage others to start the huge amount of work that will be necessary to support new ideas in anticancer drug design. However, in doing so one needs a relatively simple parent structure on which relatively simple substituents with good variation in hydrophobic, electronic and steric parameters are present when the first QSAR is established one can then make structural changes with some confidence.

Regulation of apoptosis is accomplished at multiple levels, including the initiation, transduction, amplification and execution stages and mutations that disrupt each of these stages have been detected in tumor cells.[12] Alternatively, altered expression of crucial apoptotic regulatory gene products confers different levels of resistance to apoptosis-inducing stimuli in different tumor cell lines. Because these alterations necessarily produce a selective advantage to emerging tumor cells, the identification of altered/ mutated components highlights critical regulatory points in survival and proliferation processes that merits further investigation.

Acknowledgements

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Rituximab (anti-CD20) selectively modifies Bcl-xL and apoptosis protease activating factor-1 (Apaf-1) expression and sensitizes human non-Hodgkin's lymphoma B cell lines to paclitaxel-induced apoptosis

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Abstract

The anti-CD20 monoclonal antibody rituximab (Rituxan, IDEC-C2B8) has shown promising results in the clinical treatment of a subset of patients with low grade or follicular non-Hodgkin's lymphoma (NHL). However, chemotherapy- and rituximab-refractory NHL patients may benefit from a regimen in which rituximab acts as a sensitizing agent. This study examined the apoptotic signaling mediated by rituximab on rituximab- and paclitaxel-resistant CD20$^+$ NHL B cell lines (Ramos, Raji, Daudi, and 2F7). Treatment with either rituximab (20 μg/ml) or paclitaxel (0.1–1000 nM) inhibited viable cell recovery of NHL lines. Neither rituximab nor paclitaxel induced significant apoptosis, although the combination treatment resulted in synergy in apoptosis. Rituximab selectively down-regulated Bcl-xL and induced apoptosis protease activating factor 1 (Apaf-1) expressions in Ramos cells. Paclitaxel down-regulated the expression of Bcl-xL and inhibitor of apoptosis proteins (c-IAP-1) and up-regulated the expression of Bad and Apaf-1. The combination treatment resulted in the formation of truncated Bid, cytosolic accumulation of cytochrome *c* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI, activation of caspase-9, caspase-7, caspase-3, and cleavage of poly (ADP-ribose) polymerase. The findings identify two potential novel intracellular targets of rituximab-mediated

signaling in Ramos NHL cells (*i.e.*, Bcl-xL and Apaf-1). Further, the findings show that both rituximab and paclitaxel selectively modify the expression pattern of proteins involved in the apoptosis signal transduction pathway and, through functional complementation, the combination results in synergy in apoptosis. The potential therapeutic significance of these findings is discussed. (Mol Cancer Ther. 2003;2:1183–1193)

Introduction

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of disorders that represents about 4% of all malignancies and ranks fifth in cancer incidence and mortality (1, 2). Although the initial response rates to chemotherapy are high, relapse eventually occurs and subsequent chemotherapy regimens are incapable of yielding long-term remission (3). Failure of chemotherapy to eliminate tumor cells has prompted the development of alternative therapies. A novel treatment strategy is the use of antibody (Ab)-mediated immunotherapy alone or in combination with chemotherapy.

One of the candidate antigens that has been targeted for immunotherapy is CD20, a 297-amino acid (32–37 kDa) unglycosylated phosphoprotein that spans the membrane four times (4). Although the exact function of CD20 is not yet known, it is thought to play a role in the proliferation and differentiation of B lymphocytes (4). Approximately 80–85% of NHL are B-cell malignancies in origin and >95% of these express surface CD20. CD20 is exclusively expressed in the B-cell lineage, with minimal expression on early pre-B cells and normal plasma cells. It is neither shed from the cell surface nor modulated or internalized on Ab binding (5).

The anti-CD20 monoclonal Ab (mAb) rituximab (Rituxan, IDEC-C2B8) is the first mAb approved for therapeutic use in malignancies (6). Rituximab is active as a single agent in previously treated patients with various types of lymphomas with highly favorable toxicity profile (7–9). The mechanism of action of rituximab on CD20 ligation has not been clearly delineated; however, the involvement of Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity of the malignant cells has been suggested (6, 10). Rituximab exerts considerable cytotoxicity against malignant B cells when it is hyper-cross-linked, homodimerized, or used in combination with Fc receptor-expressing accessory cells, which mimics the *in vivo* microenvironment (5, 11, 12). Rituximab also exerts a cytostatic effect on NHL cell lines *in vitro* without induction of significant apoptosis (13–15).

The naturally occurring drug paclitaxel (Taxol) is effective in the treatment of drug-refractory bladder, prostate, ovarian, and metastatic breast carcinoma and leukemia (16). In addition to the effects on microtubules and cell cycle traverse, paclitaxel can cause significant cell killing by the induction of apoptosis and necrosis depending on the cell type and concentration used (17). There is accumulating evidence that paclitaxel, either alone or in combination with other drugs such as ifosfamide, 2-chlorodeoxadenosine, or high-dose cyclophosphamide, can be used in the treatment of patients with relapsed or refractory NHL (18).

We have shown that rituximab sensitizes the 2F7 NHL cells to chemotherapeutic drugs via down-regulation of Bcl-2 (19). Paclitaxel can induce Bcl-2 phosphorylation at serine residues 70 and 87, and this has been postulated to negatively regulate the antiapoptotic effects of Bcl-2 (20). Considering that the moderate effectiveness of rituximab and paclitaxel as single agents in modulating Bcl-2 levels is well established and the fact that most NHL cells overexpress Bcl-2, we hypothesized that the combination treatment of NHL cell lines with paclitaxel and rituximab may enhance the sensitivity of these cells to cytotoxic drugs and lead to synergistic apoptosis. Noteworthy, there are no studies on the combined effects of paclitaxel and rituximab in NHL. The objectives of the present study were (1) to investigate rituximab-mediated effects on the apoptotic signaling pathway, (2) to determine whether rituximab can be used as a sensitizing agent to enhance the cytotoxic activity of paclitaxel against paclitaxel-refractory NHL cell lines, (3) to delineate the modifications of the expression pattern of gene products associated with apoptosis that are induced by each agent, and (4) to establish whether the observed synergy in apoptosis by the combination treatment correlates with complementation in gene modification by each agent.

Materials and Methods

Cell Lines and Peripheral Blood Mononuclear Cells

The Burkitt's lymphoma B cell line 2F7 (also available via American Type Culture Collection, Bethesda, MD) was isolated from a single clone of a lymph node biopsy from a patient suffering from AIDS and were provided by Dr. Otoniel Martinez-Maza (UCLA, Los Angeles, CA). The human lymphoma B cell lines Ramos (21), Raji, and Daudi were purchased from the American Type Culture Collection. The tumor cell lines were maintained in sterile 75 cm^2 tissue culture flasks in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) as described previously (19). The cell lines were maintained at a density of 0.5×10^6 cells/ml and were subcultured every 2 days.

For the generation of peripheral blood mononuclear cells (PBMC), whole blood from healthy donors was collected into 35 ml syringes with 0.5-ml sterile heparin. PBMCs were isolated by density centrifugation over a Ficoll-Hypaque density gradient (LSM, Durham, NC), washed thrice in sterile PBS, and immediately used in cytotoxicity assays.

Reagents

A stock solution of 10 mg/ml of rituximab was kept at 4°C and dilutions were prepared fresh for each experiment. Paclitaxel was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO) to make a stock solution of 6 mg/ml and was kept at room temperature. For each experiment, paclitaxel was diluted by medium to obtain the indicated concentrations. The DMSO concentration did not exceed 0.1% in any experiment. Rituximab and paclitaxel were commercially acquired.

Mouse anti-Bcl-xL mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Bad, anti-Bid, anti-caspase-9, anti-caspase-8, and anti-caspase-7 polyclonal Abs were purchased from Cell Signaling (Beverly, MA). Mouse anti-procaspase-3 and FITC-labeled anti-active caspase-3 mAbs were obtained from PharMingen (San Diego, CA). Mouse anti-actin mAb and rabbit anti-apoptosis protease activating factor 1 (Apaf-1) polyclonal Abs were purchased from Chemicon International, Inc. (Temecula, CA). Rabbit anti-survivin polyclonal Ab was purchased from Proscience Inc. (Poway, CA). Rabbit anti-inhibitors of apoptosis proteins (c-IAP-1 and c-IAP-2) and X-chromosome-linked inhibitor of apoptosis (XIAP) polyclonal Abs were purchased from Trevegen, Inc. (Gaithersburg, MD). Mouse anti-cytochrome *c* mAb and rat anti-second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) polyclonal Ab were purchased from PharMingen and Alexis Biochemicals (San Diego, CA), respectively. The Bcl-xL inhibitor 2-methoxyantimycin A3 (2MAM-A3) was purchased from Biomol (Plymouth, PA).

Rituximab Pretreatment

Ramos and 2F7 tumor cell lines (10^6 cells/ml) were grown in complete medium in 10 cm^2 tissue culture dishes (Life Technologies, Inc., Gaithersburg, MD) and were treated with a previously established optimal concentration (20 μg/ml) of rituximab for 48 h (15, 22). The cells were then washed and fresh medium was added and seeded into six-well plates (Costar, Cambridge, MA). Indicated concentrations of paclitaxel were then added, and the cells were incubated for another 16 h for maximal cytotoxicity. At the end of the incubation period, the cells were harvested and subjected to propidium iodide (PI) staining according to the specifications of the PI staining kit (Roche Diagnostics Corporation, Indianapolis, IN) and evaluated by flow cytometric analysis.

Cell Cycle Distribution and Assessment of Apoptosis by Flow Cytometric Analysis

The percentage of apoptotic cells was determined by evaluation of PI-stained preparations as described previously (5, 23) and by caspase activity (see below) of paclitaxel/rituximab-treated tumor cell lines. Cell cycle analysis and apoptosis were determined using an EpicXL flow cytometer. A minimum of 6000 events was collected on each sample and acquired in listmode by a PC Pentium computer. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content

of the intact nuclei was recorded on a logarithmic scale (5, 23). The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at the sub-G_0 phase of the cell cycle.

Caspase-3 Activity Measured by Flow Cytometric Analysis

Ramos cells (10^6 cells/ml) were grown in complete medium (control) or treated with rituximab (20 μg/ml, 48 h), paclitaxel (10 nM, 16 h), or combination of rituximab and paclitaxel. At the end of the incubation period, cells were washed once with ice-cold 1× PBS/0.1% BSA and were resuspended in 100-μl ice-cold 1× PBS/0.1% BSA. 50 μl of cell suspension (containing 2 × 10^6 cells) were aliquoted to each sample and were fixed with Cytofix/Cytoperm solution (PharMingen) for 20 min. Thereafter, the samples were washed twice with ice-cold 1× Perm/Wash buffer solution (PharMingen) and were stained with FITC-labeled anti-active caspase-3 mAb for 30 min (light protected). Thereafter, the samples were washed once with ice-cold 1× PBS/0.1% BSA followed by flow cytometric analysis. As negative control, the cells were stained with isotype control (pure IgG1) under the same conditions described above.

Cell Viability as Measured by Trypan Blue Dye Exclusion Assay

The Ramos and 2F7 cells were cultured in complete medium (control) or complete medium supplemented with rituximab, paclitaxel, and rituximab/paclitaxel under the same conditions explained above. At the end of the incubation period, the cells were harvested, a small aliquot of the cell suspension was removed and mixed with an equal volume of 0.4% trypan blue dye solution, and cell viability was determined by light microscopy. The results are representative of mean ± SD of calculated viable and dead cell numbers from three independent experiments.

Analysis of Mitochondrial Membrane Potential by 3,3′-Dihexyloxacarbocyanine Iodide Staining

Ramos cells were stained with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] to quantitate mitochondrial membrane potential. The cells were analyzed by flow cytometry with an EpicXL flow cytometer as described previously (24).

Western Blot Analysis

Tumor cells (10^7 cells/treatment) were left either untreated (control) or treated with rituximab (20 μg/ml, 48 h) or paclitaxel (10 nM, 16 h) or pretreated with rituximab (20 μg/ml, 48 h) followed by paclitaxel (10 nM, 16 h) treatment. The cells were then lysed at 4°C in radio-immunoprecipitation assay buffer as described previously (25). The cell lysates (40 μg) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad, Hercules, CA) and were subjected to Western blot analysis (25). Levels of β-actin were confirmed to ensure equal loading of the samples. The relative intensity of the bands was assessed by densitometric analysis of the digitized images and performed on an iMac computer (Apple Computer Inc., Cupertino, CA) using the public domain NIH image program (developed at NIH and also available on the Internet at http://rsb.info.nih.gov/nih-image/). Alterations of ≥40% were considered significant.

Isolation of Cytosolic Fraction and Determination of Cytochrome *c* and Smac/DIABLO Content

Ramos cells (10^7 cells/treatment) were grown under the conditions explained for Western blot. At the end of the incubation period, cells were washed twice with 1-ml ice-cold 1× PBS/0.1% BSA and were resuspended in 2 volumes of homogenization buffer (20-mM HEPES [pH 7.4], 10-mM KCl, 1.5-mM $MgCl_2$, 1-mM sodium EDTA, 1-mM sodium EGTA, 1-mM DTT, one tablet of Complete Mini protease inhibitor cocktail in 250-mM sucrose medium). After 30 min on ice, the cells were disrupted by 40 strokes of a Dounce glass homogenizer using a loose pestle (Bellco Glass, Inc., Vineland, NJ). The homogenate was centrifuged at 2500×*g* at 4°C for 5 min to remove nuclei and unbroken cells. The mitochondria were pelleted by spinning the homogenate at 16,000×*g* at 4°C for 30 min. The supernatant was removed and filtered through 0.1 μm Ultrafree MC filters (Millipore, Billerica, MA) to obtain the cytosolic fraction and was spun down at 16,000×*g* at 4°C for 15 min. The protein concentration of the supernatant was determined by the DC assay kit and was mixed with 2× Laemelli sample buffer and analyzed by SDS-PAGE for determination of cytochrome *c* and Smac/DIABLO contents in the cytosolic fraction.

Isobolographic Analysis for Determination of Synergy

Determination of the synergistic *versus* additive *versus* antagonistic cytotoxic effects of the combination treatment of the Ramos and 2F7 cell lines by rituximab and paclitaxel was assessed by isobolographic analysis as described previously (26). Briefly, isobolograms were constructed from a battery of combinations of various concentrations of rituximab (1, 10, 20, 50, 75, and 100 μg/ml) with paclitaxel (0.1–1000 nM). Combinations yielding 30 ± 5% cytotoxicity were plotted as percentage of single agent alone that resulted in the same percentage of cytotoxicity (fractional inhibitory concentration: concentration of each agent in combination/concentration of each agent alone). When the sum of this fraction (fractional inhibitory concentration) is 1, the combination is additive and the graph is geometrically expressed as a straight line; when the sum is <1, the combination is synergistic and the graph appears as concave shape; and when the sum is >1, the combination is antagonistic and the graph is geometrically represented as convex shape.

Statistical Analysis

Assays were set up in triplicates and the results were expressed as the mean ± SD. Statistical analysis and *P* value determinations were done by two-tailed paired *t* test with a confidence interval of 95% for determination of the significance of differences between the treatment groups. *P* < 0.05 was considered to be significant. ANOVA was used to test the significance among the groups. The InStat 2.01 software was used for analysis.

Results

Inhibition of Ramos and 2F7 Viable Cell Recovery by Rituximab and Paclitaxel

Flow cytometry data showed that the majority of the Ramos and 2F7 cells (>95%) express CD20 (data not shown).

Significant inhibition of viable cell recovery was observed in both cell lines on treatment with rituximab and a plateau was reached at rituximab concentrations of ≥ 20 μg/ml. Maximum inhibition of cell recovery was 32% for Ramos and 51% for 2F7 (Fig. 1A). The inhibition of cell recovery was not due to cytotoxicity as <7% of the cells were dead at 48 h of incubation (Table 1). Rituximab also inhibited cell recovery of Daudi (43%) and Raji (24%) NHL B cell lines (data not shown). Paclitaxel exerted inhibition of viable cell recovery and a plateau of ~60% of inhibition was achieved for Ramos at paclitaxel concentrations of ≥ 100 nM. For 2F7, the inhibition was more pronounced and as much as 36% inhibition was obtained at paclitaxel concentrations of ≥ 1 nM (Fig. 1B). The inhibitory effect of the combination treatment of rituximab and paclitaxel was also examined. There was no additional inhibition above that achieved by paclitaxel alone (Fig. 1C). These findings demonstrate that both rituximab and paclitaxel, alone or in combination, inhibited viable cell recovery of NHL B cell lines.
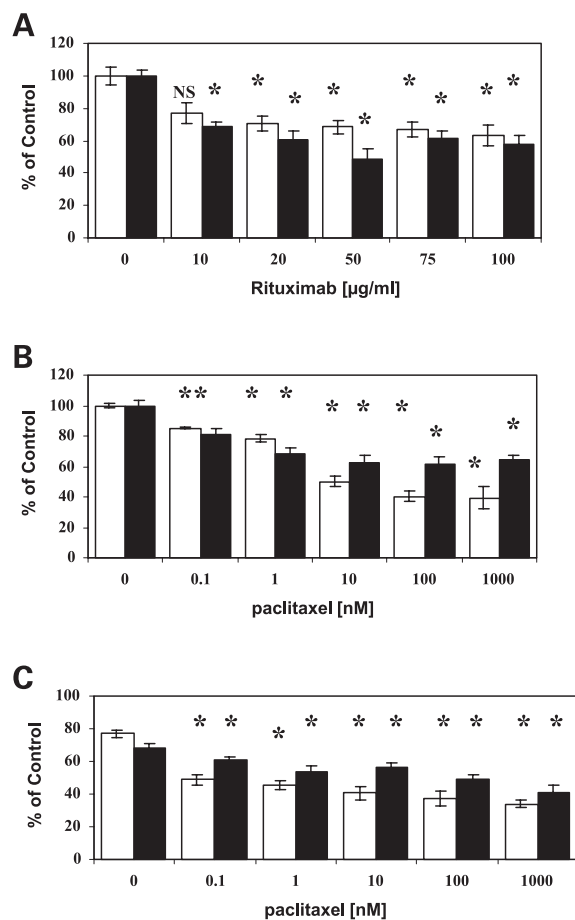
Figure 1. Cytostatic effects of rituximab, paclitaxel, and rituximab + paclitaxel on Ramos (□) and 2F7 (■) cell lines. Cells were either left untreated or treated with various concentrations of (**A**) rituximab (0 – 100 μg/ml, 48 h), (**B**) paclitaxel (0.1 – 1000 nM, 16 h), (**C**) or rituximab (20 μg/ml, 48 h) + paclitaxel (0.1 – 1000 nM, 16 h). At the end of the incubation period, the percentage of inhibition of viable cell recovery was measured microscopically by the trypan blue dye exclusion method. *Columns,* mean; *bars,* SD (*n* = 3). *, *P* < 0.01 (significant) compared with control.

Effects of Rituximab, Paclitaxel, and Combination on Cell Cycle

Rituximab and paclitaxel, alone and in combination, were evaluated for their effects on the cell cycle distribution. The findings are summarized in Fig. 2 and Table 1. The Ramos and 2F7 cells were treated with rituximab (20 μg/ml, 48 h) and paclitaxel (1 and 10 nM, 16 h). The results of three independent experiments demonstrate that rituximab has no effect on the cell cycle distribution of Ramos and 2F7 cells. Paclitaxel induced significant arrest of both cell lines at the G_2-M phase of the cell cycle and the extent of cell cycle arrest inversely correlated with the paclitaxel concentration used. The combination treatment-pretreatment with rituximab for 48 h followed by paclitaxel for 16 h showed less cells arrested at G_2-M.

Synergy Is Achieved for Apoptosis by Combination Treatment of Ramos Cells with Paclitaxel and Rituximab

We examined whether the sensitizing effect of rituximab to paclitaxel-mediated cytotoxicity was synergistic (26). Flow cytometric analysis, as illustrated in Fig. 2A, clearly demonstrates that rituximab treatment alone does not induce apoptosis in Ramos cells. Paclitaxel, however, induced moderate apoptosis at 1 nM, which was not potentiated by 10-fold increase (Table 1; Fig. 2A). In contrast, the combination of rituximab (20 μg/ml) and paclitaxel (1 nM, 10 nM) resulted in significant potentiation of apoptosis as shown by the accumulation of the hypodiploid cells at the sub-G_0 phase of the cell cycle (Fig. 3A; Table 1). At the 1 and 10 nM concentrations of paclitaxel, >35% and >43% of the cells underwent apoptosis, respectively. A representative experiment is depicted in Fig. 2A and the mean of three experiments is shown in Table 1. The observed augmentation of apoptosis by the combination treatment with rituximab and paclitaxel resulted in synergistic apoptosis as determined by isobolographic analysis (Fig. 3). Time kinetics studies demonstrated that induction of apoptosis by the combination treatment started at 8–12 h post-treatment and reached the maximum levels by 16 h. Prolongation of the incubation period did not enhance the level of apoptosis (data not shown).

To validate the PI staining and sub-G_0 hypodiploid cell population as the true representatives of apoptosis (5, 23), the Ramos cells were treated under the conditions mentioned above. The samples were divided into two equal proportions. One proportion was subjected to PI staining and DNA fragmentation analysis and the other half was stained with FITC-labeled anti-active caspase-3 mAb. A close correlation was established between the percentage of hypodiploid cells accumulating at the sub-G_0 region with those possessing active caspase-3 (Table 2). Collectively, these results validate the DNA fragmentation assay (PI) for the measurement of apoptosis.

Under the same conditions, 2F7 cells were examined for synergy by the combination treatment. No synergy in apoptosis was achieved by the combination of rituximab and paclitaxel (Fig. 3B). The failure to detect sensitization by rituximab to paclitaxel-mediated apoptosis (Fig. 2B) was

Table 1. Cell cycle analysis and apoptosis by rituximab, paclitaxel, and combination treatment of Ramos and 2F7 NHL cell lines

| Treatment	G_0-G_1	S	G_2-M	Apoptosis
Ramos				
Control	42.0 ± 6.2	25.7 ± 2.9	27.1 ± 3.8	3.8 ± 0.7
DMSO	38.3 ± 3.7	28.1 ± 4.1	26.1 ± 6.5	6.0 ± 2.2
Rituximab (20 μg/ml)	37.5 ± 5.1	28.0 ± 4.1	30.3 ± 5.2	2.7 ± 0.6
Paclitaxel (1 nM)	16.8 ± 0.4	9.2 ± 1.3	60 ± 1.5	13.3 ± 1.8
Paclitaxel (10 nM)	26.4 ± 2.8	20.1 ± 8.5	39.9 ± 6	12.4 ± 0.8
Rituximab (20 μg/ml) + paclitaxel (1 nM)	24.3 ± 4.1	19.3 ± 0.9	22.5 ± 0.7	35.5 ± 5.1[a]
Rituximab (20 μg/ml) + paclitaxel (10 nM)	19.6 ± 3.2	16.3 ± 3.9	21.4 ± 2.9	45.3 ± 2.5[a]
2F7				
Control	48.8 ± 6.3	20.4 ± 1.1	23.5 ± 6.0	6.5 ± 1.7
DMSO	49.4 ± 1.4	20.1 ± 0.5	22.5 ± 2	7.2 ± 1.5
Rituximab (20 μg/ml)	54.6 ± 1.6	18.6 ± 0.6	18.1 ± 1.8	7.9 ± 0.6
Paclitaxel (1 nM)	22.1 ± 2.2	9.8 ± 2.2	46 ± 1.9	21.2 ± 2.4
Paclitaxel (10 nM)	18.1 ± 1.6	14.4 ± 3.2	48.1 ± 3.8	18.7 ± 1.9
Rituximab (20 μg/ml) + paclitaxel (1 nM)	23.7 ± 3.6	7.5 ± 2.2	46.2 ± 3.8	22.3 ± 2.4[b]
Rituximab (20 μg/ml) + paclitaxel (10 nM)	29.8 ± 7.3	15.2 ± 4.9	42.9 ± 1	10.8 ± 1.3[b]

Note: The cells (Ramos and 2F7) were either left untreated (control) or pretreated with rituximab (20 μg/ml, 48 h). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with various concentrations of paclitaxel (1 and 10 nM, 16 h). At the end of the incubation period, the cells were stained with PI solution and cell cycle analysis was assessed by flow cytometry. The percent apoptosis was determined as the percentage of hypodiploid cells accumulated at the sub-G_0 phase of the cell cycle. The results are represented as mean $\pm$ SD ($n = 3$).
[a]$P < 0.001$ (very significant).
[b]Not significant compared with paclitaxel treatment alone.
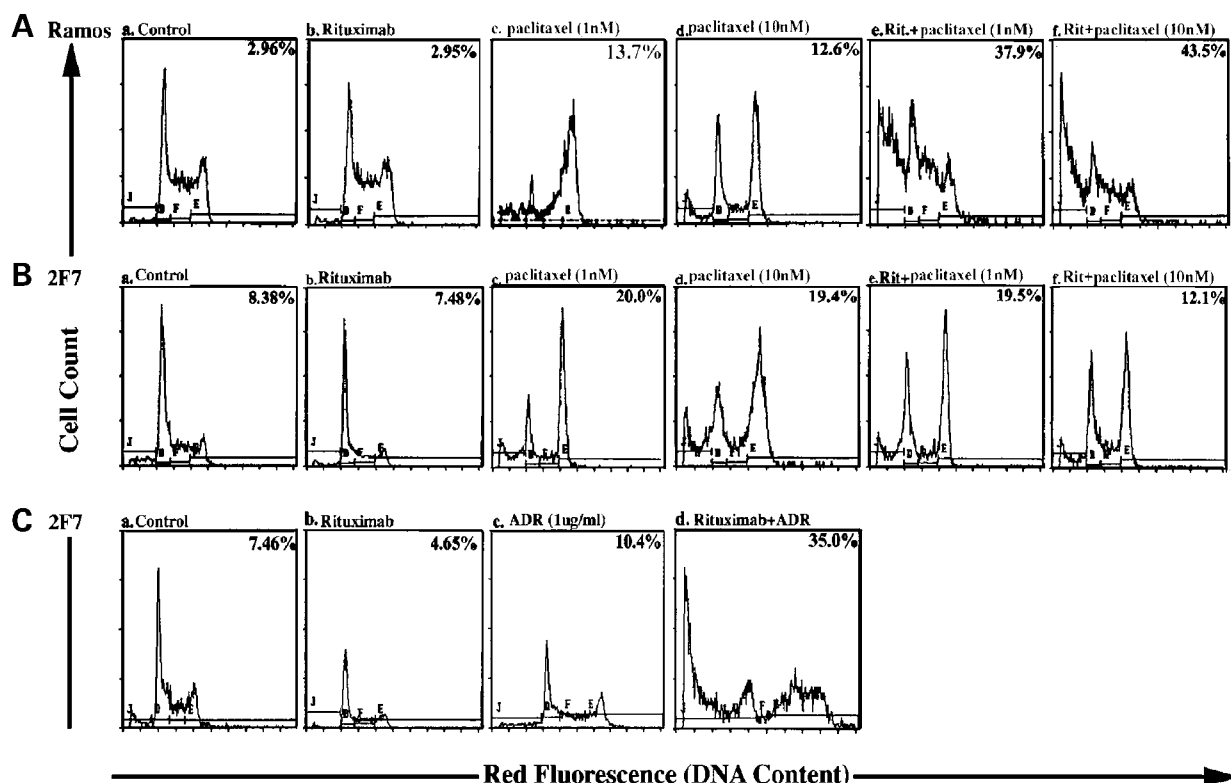
Figure 2. Sensitization of Ramos and 2F7 cell lines by rituximab to paclitaxel and ADR-mediated apoptosis. Ramos and 2F7 cells were either left untreated or pretreated with rituximab (20 μg/ml, 48 h). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with various concentrations of (**A** and **B**) paclitaxel (1 and 10 nM, 16 h) or (**C**) ADR (1 μg/ml) was added to the rituximab-pretreated 2F7 cells for an additional 16 h. Then, the cells were stained with PI solution (DNA fragmentation assay) and analyzed by flow cytometry. The percentage of apoptotic cells (sub-G_0 population) is indicated at the upper right corner of each panel. The findings demonstrate that rituximab sensitizes Ramos and not 2F7 to paclitaxel-mediated apoptosis. However, rituximab sensitizes 2F7 to ADR-mediated apoptosis. The results are representative of three independent experiments.

A

B
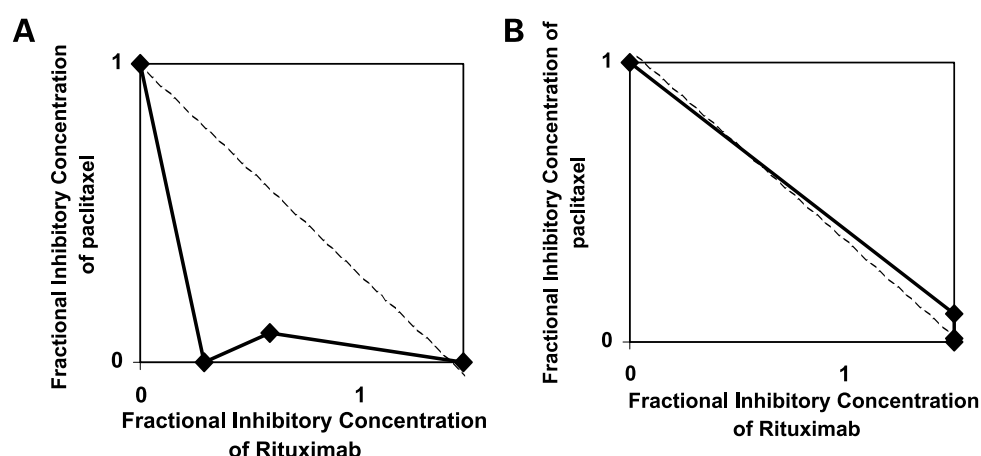
Figure 3. Isobolographic analysis for the determination of synergistic effects of rituximab/paclitaxel combination treatment on Ramos (**A**) and 2F7 (**B**) cell lines. The cells were treated under the conditions explained in Fig. 2, and the synergistic *versus* additive *versus* antagonistic effects of rituximab/paclitaxel treatment of both cell lines was evaluated by isobolographic analysis as described previously (26). Synergy is shown for Ramos and not for 2F7 cells.

not due to the inherent resistance of 2F7 cells to the sensitizing effects of rituximab. Treatment of the 2F7 cells with rituximab sensitized the cells to Adriamycin (ADR)-induced apoptosis (Fig. 2C).

Rituximab-mediated sensitization to paclitaxel-induced apoptosis was not limited to the Ramos cells. Pretreatment of other NHL cells such as Raji and Daudi with rituximab rendered them sensitive to apoptosis induced by paclitaxel (Table 3). Noteworthy, the combination of rituximab and paclitaxel was not toxic and did not potentiate the killing of freshly derived human PBMCs beyond the background levels (Fig. 4). At 10 nM paclitaxel, rituximab-pretreated PBMCs exhibited $9 \pm 1.6\%$ apoptosis compared with rituximab ($6.0 \pm 1.8\%$) or paclitaxel ($5.9 \pm 1.1\%$) treatment alone.

The above data demonstrate that rituximab is capable of sensitizing paclitaxel-resistant NHL B cell lines to paclitaxel-induced apoptosis, whereas minimal toxicity is observed by the combination on PBMC.

Effects of Rituximab and Paclitaxel, Alone and in Combination, on the Expression of Apoptotic Regulatory Proteins in Ramos Cells

We have chosen Ramos cells as the model to examine the mechanism of sensitization by rituximab to paclitaxel mediated apoptosis. We have postulated that signaling may result from alterations in the expression levels of a number of proapoptotic as well as antiapoptotic gene products on rituximab and paclitaxel treatments. Rituximab treatment of Ramos cells resulted in down-regulation of Bcl-xL expression and up-regulation of Apaf-1. Rituximab did not regulate the expression of several other apoptotic gene products examined (Fig. 5, A and E). Treament of Ramos cells with paclitaxel resulted in down-regulation of the antiapoptotic proteins Bcl-xL and c-IAP-1. Paclitaxel up-regulated the expression of proapoptotic Bad and significantly induced Apaf-1 expression (Fig. 5, A and E). Combination of rituximab and paclitaxel resulted in complete abrogation of Bcl-xL expression and pronounced decrease in the expression of survivin, c-IAP-1, and c-IAP-2. However, the expression level of XIAP was not significantly altered by the combination treatment (Fig. 5, A and D). In addition, the combination treatment resulted in the cleavage of Bid (truncated Bid [tBid]), another proapoptotic member of the Bcl-2 family, which migrates to the mitochondria and ensures mitochondrial destabilization.

Neither rituximab nor paclitaxel significantly activated caspase-3, caspase-7, or caspase-9 (Table 2; Fig. 5B). These caspases were activated by the combination treatment, leading to the cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 5B). The instability of mitochondria was further enhanced by significant decrease in mitochondrial transmembrane potential ($\Delta\psi$m) by the combination of

Table 2. Comparison of apoptosis measured by PI staining and caspase-3 activation

	% Apoptosis			
	Control	Rituximab (20 μg/ml)	Paclitaxel (10 nM)	Rituximab + paclitaxel
PI staining	1.32 ± 0.26	1.85 ± 0.8	12.08 ± 1.4	46.9 ± 1.9
Caspase-3 activation	5.34 ± 1.82	9.7 ± 2.8	10.9 ± 12.2	46.7 ± 3.3

Note: The Ramos cells were treated under the same conditions explained in Table 1. Thereafter, the samples were divided in two equal proportions. One half was subjected to PI staining and the percent apoptosis was measured by the percent of hypodiploid cells accumulated at sub-G_0 phase of the cell cycle. The second half was subjected to staining with FITC-labeled anti-active caspase-3 mAb and fluorescence-activated cell sorting analysis. Samples were set up in duplicates and the results are represented as means $\pm$ SEM ($n = 2$).
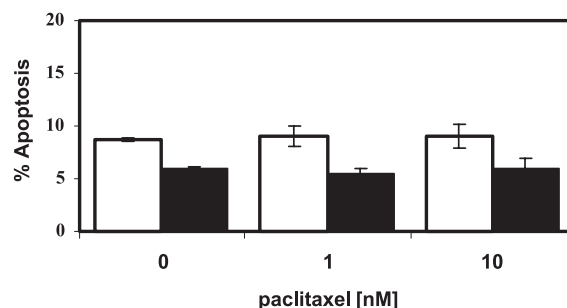
paclitaxel and rituximab (Table 4; Fig. 5D). Further analysis revealed significant accumulation of cytochrome *c* and Smac/DIABLO in the cytosolic fraction (Fig. 5, C and E) by the combination treatment. Cytosolic accumulation of these proteins paralleled their depletion from mitochondrial fraction (data not shown), which confirms their redistribution from the mitochondria to the cytosol.

Collectively, these results demonstrate that rituximab and paclitaxel selectively inhibit the expression of Bcl-xL and up-regulate the expression of Apaf-1 in Ramos cells. In addition, paclitaxel up-regulates Bid and inhibits c-IAP-1 expression. Further, the findings demonstrate that each agent, by itself, was insufficient for the full activation of the mitochondrial pathway for apoptosis. However, the combination of rituximab and paclitaxel, by functional complementation, activated the mitochondrial pathway and facilitated the apoptotic signal to fully proceed toward apoptosis.

Role of Bcl-xL Expression in Resistance to Paclitaxel-Induced Apoptosis

The above findings demonstrate that rituximab selectively down-regulates the expression of antiapoptotic Bcl-xL in Ramos cells, which might be implicated as pivotal to maintain resistance to paclitaxel. Accordingly, inhibition of Bcl-xL activity should mimic rituximab effects and sensitize the cells to paclitaxel-induced apoptosis. This was tested by the use of a specific inhibitor. 2MAM-A3 has protein binding activity and binds to the hydrophobic groove bounded by the BH1, BH2, and BH3 domains on the surface of Bcl-xL (27), thus preventing its dimerization with the proapoptotic Bcl-2 family members. This will alter the proapoptotic/antiapoptotic ratio and favor the apoptotic signaling to proceed.

To ascertain the protective role of Bcl-xL in Bcl-2-deficient Ramos cells, the cells were either left untreated or pretreated with rituximab (20 µg/ml, 48 h) or 2MAM-A3 (15 µg/ml, 6 h). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with paclitaxel (10 nM, 16 h). At the end of the incubation period, the cells were subjected to PI staining (DNA fragmentation assay) and flow cytometric analysis. 2MAM-A3 induced

Table 3. Rituximab-mediated sensitization of NHL cell lines to paclitaxel-induced apoptosis

	Paclitaxel (nM)			
	Control	0.1	1	10
Raji	3.0 ± 2.8	6.5 ± 1.1	17.7 ± 2.1	19.8 ± 1.6
Raji + rituximab	4.8 ± 1.2	12.2 ± 1.3^a	28.8 ± 1.6^b	31.2 ± 1.5^b
Daudi	2.4 ± 1.1	3.8 ± 2.1	6.3 ± 0.7	11.6 ± 1.4
Daudi + rituximab	5.9 ± 3.2	13.3 ± 1.9^b	18.7 ± 1.6^c	23.5 ± 1.7^b

Note: Raji and Daudi cells were treated under the conditions explained in Table 1 and the percentage of apoptosis was determined by flow cytometry. Samples were set up in duplicates and the data are represented as means $\pm$ SD (n = 2).
[a]Not significant compared with paclitaxel treatment alone.
[b]$P < 0.01$ (significant).
[c]$P < 0.001$ (very significant).

Figure 4. Failure of rituximab to sensitize PBMCs to paclitaxel-mediated apoptosis. PBMCs (10^6 cells/ml) were either left untreated (□) or pretreated with rituximab (■; 20 µg/ml, 48 h). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with various concentrations of paclitaxel (1 and 10 nM, 16 h). Then, the cells were stained with PI solution (DNA fragmentation assay) and analyzed by flow cytometry. *Columns,* mean; *bars,* SD (n = 2). The data show that the combination treatment was not toxic to PBMC.

modest apoptosis in Ramos cells ($9.1 \pm 1.5\%$). However, it sensitized the cells to paclitaxel-induced apoptosis at levels comparable with that achieved by rituximab pretreatment ($37.1 \pm 1.8\%$; $P < 0.05$, compared with paclitaxel alone). Similar results were obtained with other cell lines (data not shown). These findings suggest that diminished expression (by rituximab) and functional impairment (by 2MAM-A3) of Bcl-xL is sufficient to overcome paclitaxel resistance.

Discussion

The present study provides evidence for the first time that treatment of refractory NHL cell lines with rituximab potentiates the cytotoxic effect of paclitaxel and leads to synergy in apoptosis. The observed synergy in apoptosis achieved by the combination of rituximab and paclitaxel appears to be the result of complementation by the selective modification of apoptosis regulatory proteins by each agent alone. Rituximab selectively down-regulated the expression of antiapoptotic Bcl-xL and up-regulated the expression of proapoptotic Apaf-1 in Ramos cells. These modifications along with those exerted by paclitaxel were presumably sufficient to avert the resistance to apoptosis of paclitaxel-refractory NHL cells. Hence, the combination treatment resulted in the destabilization of the mitochondria, release of cytochrome *c* and Smac/DIABLO, activation of caspase-3, caspase-7, and caspase-9, subsequent cleavage of caspase substrates, and apoptosis. These data demonstrate that the combination of rituximab and subtoxic concentrations of paclitaxel, both used at clinically achievable concentrations (16, 22), may be effectively used against paclitaxel-refractory NHL.

It is well documented that paclitaxel inhibits microtubule depolymerizarion and promotes the formation of metastable microtubules. This interferes with the normal function of microtubules, prevention of mitotic spindle formation, and subsequent arrest of the cell cycle progression at the late G_2-M phases (16). In agreement, paclitaxel caused G_2-M arrest of all of the four NHL B cell lines used in the study, albeit with varying degrees.

Figure 5. Western blot analysis for detection of alterations in protein expression by rituximab, paclitaxel, or combination. **A,** modifications of apoptotic gene products; **B,** activation of caspases and PARP cleavage; **C,** cytosolic accumulation of cytochrome *c* and Smac/DIABLO; **D,** flow cytometry histograms demonstrating alterations in $\Delta\psi_m$; **E,** densitometric analysis and the relative intensity of the modulation of apoptosis regulatory gene products. Ramos cells were grown in (*1*) the absence (complete medium control), (*2*) the presence of rituximab (20 μg/ml, 48 h), (*3*) the presence of paclitaxel (10 nM, 16 h), or (*4*) pretreatment with rituximab (20 μg/ml, 48 h) + paclitaxel (10 nM, 16 h). Total cell lysates [C: cytosolic fractions] (40 μg) were subjected to Western blot analysis as described in "Materials and Methods." Relative intensity of the bands and statistical significance were assessed as described in "Materials and Methods." Analysis of $\Delta\psi_m$ was performed as detailed in Table 4. *Arrows,* cleaved form of the proteins. *Columns,* mean; *bars,* SD (*n* = 2).

Table 4. Alterations in $\Delta\psi$m by the combination of rituximab and paclitaxel

Control	% Alterations in $\Delta\psi$m		
	Rituximab (20 µg/ml)	Paclitaxel (10 nM)	Rituximab + paclitaxel
9.6 ± 2.8	11.7 ± 1.3	14.8 ± 2.9	53.8 ± 4.8

Note: The Ramos cells were treated under the same conditions explained in Table 1. After the incubation period, the cells were washed, stained with DiOC$_6$(3) for 30 min at 37°C, and analyzed by flow cytometry. The samples were set up in duplicates and the results are presented as means ± SD of percentage of the cells with depolarized mitochondria ($n = 2$).

Rituximab treatment of NHL cells neither induced perturbations in cell cycle distribution nor induced significant cytotoxicity in NHL cell lines. In contrast to mouse anti-CD20 mAbs such as 1F5 that stimulates cell cycle transition from G_0 to G_1 or B1, which inhibits B-cell progression from G_1 to S-G_2-M phase of the cell cycle (4, 5), the antihuman CD20 mAb rituximab inhibits cellular proliferation with no apparent effects on any specific phase of the cell cycle (19). Rituximab alone does not induce apoptosis, whereas previous findings showed that hyper-cross-linking (11) or homodimers (12) of rituximab are capable of inducing apoptosis. The failure of rituximab to induce apoptosis in this study can be explained by the usage of monomeric (non-cross-linked) rituximab.

Rituximab pretreatment sensitized Ramos cells to paclitaxel-mediated apoptosis (Table 1; Fig. 2A) in a synergistic manner (Fig. 3A). Rituximab also sensitized additional NHL cell lines to paclitaxel-induced apoptosis (Table 3). In contrast, rituximab failed to sensitize the 2F7 cells to paclitaxel-induced apoptosis (Fig. 2B; Table 1). This failure is not due to an inherent inability of rituximab to sensitize the 2F7 cells to chemotherapeutic drugs because rituximab sensitized the 2F7 cells to apoptosis induced by ADR (Fig. 2C) and other drugs (15).

To delineate the potential underlying molecular mechanism of the observed synergy in apoptosis in Ramos cells, we present evidence that both rituximab and paclitaxel down-regulated the expression of Bcl-xL at the protein level (Fig. 5A). While we have previously demonstrated that rituximab down-regulates Bcl-2 in 2F7 (19), in this study, however, we demonstrate that in Bcl-2-deficient Ramos cells (11) the Bcl-2 homologue, Bcl-xL, is a novel intracellular target of rituximab. Rituximab-induced down-regulation of Bcl-xL was also noticed in other NHL B cell lines studied (Raji and Daudi; data not shown). The mechanism by which rituximab inhibits Bcl-xL is not known. Preliminary findings suggest that rituximab inhibits activator protein-1 (AP-1) activity, which has been shown to regulate Bcl-xL expression (28). Paclitaxel also down-regulated Bcl-xL expression, which is in agreement with previously reported data (29). Accumulating evidence suggests a regulatory role of Bcl-xL in the paclitaxel signal transduction pathway. Bcl-xL-expressing ovarian carcinoma and hepatoblastoma HepG2 cells exhibited high

resistance to paclitaxel and other drugs (30, 31). Ectopic overexpression of Bcl-xL blocks paclitaxel, etoposide, ADR, and camptothecin induced apoptosis in HL-60, NIH3T3 fibroblasts, and IL-3-dependent murine myeloid 32D cells (32). Our findings are in agreement with these results. Further, previous findings have shown that functional impairment of Bcl-xL in cells expressing high levels of Bcl-xL can overcome the drug resistance and induce apoptosis (27). Our findings suggest that decreased expression and functional impairment of Bcl-xL by rituximab (Fig. 5A) and 2MAM-A3, respectively, are sufficient to overcome paclitaxel resistance in Ramos NHL cells. Further, these findings suggest the role of Bcl-xL as a resistant factor and suggest that the inhibition of Bcl-xL expression by rituximab in Ramos cells is responsible for sensitization to paclitaxel-induced apoptosis.

Slight induction of Bad was observed on paclitaxel treatment of Ramos cells (Fig. 5A). Bad has been shown to partly account for paclitaxel resistance of ovarian carcinoma cells (33). Because the ratio between death repressors and death promoters of the Bcl-2 family members is a key determinant of the cellular fate in response to noxious stimuli, our results suggest that concurrent decrease in Bcl-xL and increase in Bad protein levels will favor the apoptosis signal to proceed.

Significant up-regulation of Apaf-1 expression by rituximab and paclitaxel was observed (Fig. 5A). Recently, we have reported that up-regulation of Apaf-1 by ADR might be implicated in the sensitization of ADR-resistant human multiple myeloma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (25). Enforced overexpression of ectopic Apaf-1 decreased the threshold of apoptosis of HL-60 cells in response to etoposide and paclitaxel (24, 34), which was inhibited in cells with high levels of Bcl-2 or Bcl-xL (31). Rituximab, via direct or indirect DNA damage, in a p53-dependent manner (35), may up-regulate Apaf-1. Rituximab may also increase the protein stability of Apaf-1 via a proteosome-dependent pathway.

We have also observed slight down-regulation of c-IAP-1 by paclitaxel. IAP family members (c-IAP-1, c-IAP-2, XIAP, and survivin) selectively suppress different apoptotic pathways initiated by stimuli that release cytochrome *c* from mitochondria. IAPs inhibit these pathways through the binding to and ablating of the proteolytic processing of distinct caspases that function in the distal portions of the proteolytic cascades involved in apoptosis such as caspase-3, caspase-6, caspase-7, and caspase-9 but not the upstream initiator caspase-8 (36–38)

Combination of rituximab and paclitaxel resulted in total loss of Bcl-xL and pronounced down-regulation of survivin and both c-IAP-1 and c-IAP-2. IAP family members are expressed in a large panel of tumors from various origins including NHL while undetectable in normal adult tissues (36–38). Because most chemotherapeutic agents exert their effects via the mitochondrial pathway (type II) and the fact that IAPs do not bind to caspase-8, the expression of IAPs might reflect an additional level of protection of NHL cells
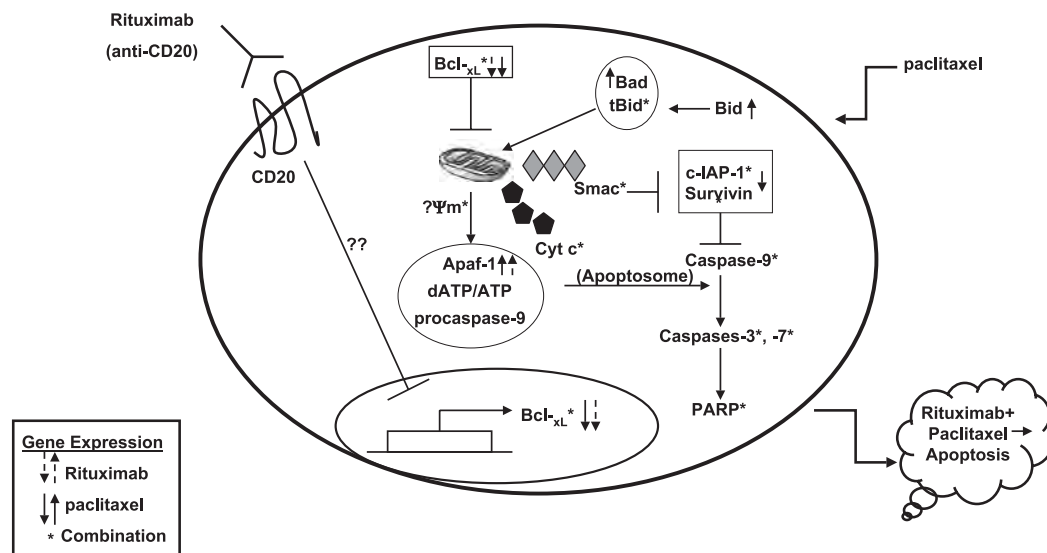
Figure 6. Proposed model of rituximab-mediated sensitization of Ramos cells to paclitaxel-induced apoptosis. On ligation to the B-cell restricted marker CD20, rituximab interferes with apoptosis signaling pathways via regulation of protein expression. The molecular events triggered by rituximab include decrease in the expression of the antiapoptotic protein Bcl-xL and the induction of the proapoptotic Apaf-1. Paclitaxel down-regulates the antiapoptotic proteins Bcl-xL and c-IAP-1 and up-regulates the expression of proapoptotic Bid and Apaf-1. Yet, these various modulatory effects by themselves are inadequate for the full induction of apoptosis. The combination treatment, via functional complementation, results in the formation of proapoptotic tBid and the induction of apoptosis. tBid migrates to and reside in the mitochondrial outer membrane. Decreased levels of Bcl-xL by rituximab and paclitaxel and the presence of tBid and high levels of Bad will alter the ratio of proapoptotic/antiapoptotic Bcl-2 family members. Decrease in this ratio, which is a key determinant in cellular fate in response to noxious stimuli, will collapse the $\Delta\psi_m$ and facilitate the release of apoptogenic molecules such as cytochrome *c* and Smac/DIABLO. Smac/DIABLO will bind to and repress the inhibitory effects of IAPs. Increased levels of Apaf-1 in combination with cytochrome *c* and dATP/ATP will facilitate the assembly of the apoptosome complex. Through autocatalytic processing, procaspase-9 becomes activated concurrently with decreased levels of certain IAPs and caspase-9 will activate caspase-3 and caspase-7 to subsequently cleave PARP and induce apoptosis. *Solid and dashed arrows,* the signaling molecules altered by paclitaxel and rituximab, respectively. *, Modulation by the combination treatment.

against chemotherapy (36–38). Therefore, modulation of the expression of IAPs shown here will decrease the apoptosis threshold and might contribute to the enhanced drug sensitivity of the NHL cells.

The combination treatment also resulted in the formation of tBid. When there is a block in the receptor-mediated signaling pathway (type I), small amounts of caspase-8 will cleave Bid. tBid will then migrate to and reside in the mitochondrial outer membrane, which will act as an amplification loop (39) for the induction of apoptosis. Thus, induction of Bad, formation of tBid (proapoptotic molecules), concurrence with the absence of Bcl-2, and complete abrogation of Bcl-xL (antiapoptotic molecules) will destabilize mitochondria. This notion is further supported by the observation that combination treatment resulted in decrease in $\Delta\psi_m$ (Table 4; Fig. 5D), cytosolic accumulation of cytochrome *c* and Smac/DIABLO (Fig. 5, C and E), subsequent activation of caspase-9, caspase-7, caspase-3, and PARP cleavage. Altogether, these findings favor the employment of the type II mitochondrial signaling pathway for the induction of apoptosis (40). Thus, the effects of rituximab and paclitaxel on the apoptotic signal transduction pathway suggest that each agent selectively modifies certain apoptotic gene products. Hence, rituximab complements and facilitates the cytotoxic activity of paclitaxel and the combination will result in the execution of apoptosis. The complementation model is schematically represented in Fig. 6.

The present findings emphasize the value of the complementation approach (Fig. 6) in the treatment of rituximab/drug-resistant NHL tumors cells. We suggest that the combination of a nontoxic agent such as rituximab and subtoxic concentrations of a chemotherapeutic drug such as paclitaxel, via selective regulation of expression of apoptosis-associated proteins, results in the reversal of resistance via synergy in apoptosis.

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Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-κB and inhibition of Bcl-$_{xL}$ expression

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues and this prompted its potential therapeutic application in cancer. However, not all cancers are sensitive to TRAIL-mediated apoptosis and, therefore, TRAIL-resistant cancer cells must be sensitized first to become sensitive to TRAIL. Treatment of prostate cancer (CaP) cell lines (DU145, PC-3, CL-1, and LNCaP) with nitric oxide donors (e.g. (Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate (DETA-NONOate)) sensitized CaP cells to TRAIL-induced apoptosis and synergy was achieved. The mechanism by which DETANONOate mediated the sensitization was examined. DETANONOate inhibited the constitutive NF-κB activity as assessed by EMSA. Also, p50 was S-nitrosylated by DETANONOate resulting in inhibition of NF-κB. Inhibition of NF-κB activity by the chemical inhibitor Bay 11-7085, like DETANONOate, sensitized CaP to TRAIL apoptosis. In addition, DETANONOate downregulated the expression of Bcl-2 related gene (Bcl-$_{xL}$) which is under the transcriptional regulation of NF-κB. The regulation of NF-κB and Bcl-$_{xL}$ by DETANONOate was corroborated by the use of Bcl-$_{xL}$ and Bcl-x κB reporter systems. DETANONOate inhibited luciferase activity in the wild type and had no effect on the mutant cells. Inhibition of NF-κB resulted in downregulation of Bcl-$_{xL}$ expression and sensitized CaP to TRAIL-induced apoptosis. The role of Bcl-$_{xL}$ in the regulation of TRAIL apoptosis was corroborated by inhibiting Bcl-$_{xL}$ function by the chemical inhibitor 2-methoxyantimycin A$_3$ and this resulted in sensitization of the cells to TRAIL apoptosis. Signaling by DETA-NONOate and TRAIL for apoptosis was examined. DETANONOate altered the mitochondria by inducing membrane depolarization and releasing modest amounts of cytochrome c and Smac/DIABLO in the absence of downstream activation of caspases 9 and 3. However, the combination of DETANONOate and TRAIL resulted in activation of the mitochondrial pathway and activation of caspases 9 and 3, and induction of apoptosis. These findings demonstrate that DETANONOate-mediated sensitization of CaP to TRAIL-induced apoptosis is via inhibition of constitutive NF-κB activity and Bcl-$_{xL}$ expression.

Introduction

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutics such as drugs, irradiation, and immunotherapy since most of their primary cytotoxic effects are through apoptosis (Ng and Bonavida, 2002a; Hersey and Zhang, 2003). Therefore, after the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, antitumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (Ashkenazi and Dixit, 1999; Ashkenazi *et al.*, 1999). TRAIL has been considered a new therapeutic, and preclinical studies demonstrate its antitumor activity alone or in combination with drugs (Ashkenazi *et al.*, 1999; De Jong *et al.*, 2001; Wajant *et al.*, 2002; Chawla-Sarkar *et al.*, 2003). However, many tumor cells have been shown to be resistant to TRAIL (Zisman *et al.*, 2001; Ng *et al.*, 2002; Bouralexis *et al.*, 2003; Tillman *et al.*, 2003). We and others have reported that various sensitizing agents like chemotherapeutic drugs (Zisman *et al.*, 2001; Munshi *et al.*, 2002), cytokines (Park *et al.*, 2002), and inhibitors (Nyormoi *et al.*, 2003) are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

Prostate cancer (CaP) cells have been shown to exhibit constitutive nuclear factor κB (NF-κB) activity (Suh *et al.*, 2002). It has been recently reported that NF-κB can regulate the sensitivity of target cells to TRAIL apoptosis in hepatoma cells (Shigero *et al.*, 2003). In addition, it has been reported that CaP cells overexpress Bcl-2 related gene (Bcl-xL), which negatively regulates tumor cells sensitivity to drug-mediated apoptosis (Raffo *et al.*, 1995). Studies on Bcl-xL gene transcription demonstrate that Bcl-xL is regulated in part by NF-κB (Mori *et al.*, 2001). Thus, constitutive expression of NF-κB in CaP may regulate the constitutive expression of Bcl-xL. We have reported that nitric oxide (NO) donors can sensitize tumor cells to FasL and tumor necrosis factor alpha (TNF-α)-mediated apoptosis (Garban and Bonavida, 2001a, b). Further, we (Huerta-Yepez *et al.*, 2003) and others (Lee *et al.*, 2001; Secchiero *et al.*, 2001) reported that (Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate (DETANONO-ate) can also sensitize tumor cells to TRAIL-mediated apoptosis.

The mechanism underlying the NO-mediated sensitization to TRAIL is not known. We hypothesized that NO-mediated sensitization of CaP cells to apoptosis may be due to NO-induced inhibition of constitutive NF-κB activity and this, in turn, will result in the downregulation of Bcl-xL transcription and expression. Hence, downregulation of the antiapoptotic gene product Bcl-xL will result in the sensitization of CaP cells to TRAIL-mediated apoptosis. This study was designed to test this hypothesis and the followings were investigated: (1) Does NO sensitize androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis? (2) Does NO inhibit constitutive NF-κB activity resulting in inhibition of Bcl-xL expression? (3) Do inhibitors of NF-κB and Bcl-xL mimic NO and sensitize CaP to TRAIL-mediated apoptosis? And (4) by what mechanism does NO modify the apoptotic signaling pathway and sensitize CaP to TRAIL-mediated apoptosis?

Results

Sensitization of CaP cell lines to TRAIL-mediated apoptosis by DETANONOate

Our previous findings have demonstrated that CaP cell lines (LNCaP, DU-145, PC-3, and CL-1) are relatively resistant to TRAIL-mediated apoptosis (Zisman *et al.*, 2001; Ng *et al.*, 2002), and are shown in Figure 1a. However, pretreatment of CaP cell lines with the NO donor DETANONOate resulted in significant potentiation of apoptosis by TRAIL for the four cell lines tested. The extent of potentiation was a function of the concentration of TRAIL used (Figure 1a). The sensitization by DETANONOate was synergistic as determined by isobologram analysis (Figure 1b). We selected PC-3 as a model system for further investigation. Treatment of PC-3 cells with various concentrations of DETANONOate sensitized the cells to TRAIL-induced

apoptosis, and the extent of apoptosis was a function of the concentration of DETANONOate used (Figure 1c). In addition to apoptosis, NO, TRAIL, and the combination inhibited cell proliferation significantly (Figure 1d). These findings demonstrate that DETA-NONOate sensitizes androgen-dependent and -independent CaP tumor cell lines to TRAIL-mediated apoptosis and synergy is achieved. Previous findings demonstrated that the androgen 5-α dihydrotestosterone (DHT) sensitizes LNCaP to 12-O-tetradecanoylphorbolacetate (TPA)-induced apoptosis (Altuwaijri *et al.*, 2003). We examined whether DHT also sensitizes LNCaP to TRAIL apoptosis. We observed that treatment of LNCaP with DHT sensitizes the cells to TRAIL (Table 1).

DETANONOate inhibits NF-κB activity and inhibition of NF-κB sensitizes PC-3 to TRAIL apoptosis

We examined the effect of DETANONOate on NF-κB activity in PC-3 cells. The cells were treated with DETANONOate (500 and 1000 μM) and tested for NF-κB activity by EMSA. In addition, we used the NF-κB inhibitor, Bay 11-7085, at different concentrations as control for inhibition of NF-κB activity. Figure 2a demonstrates that DETANONOate inhibits NF-κB activity significantly and the inhibition at 1000 μM was much higher than the inhibition at 500 μM. As expected, the Bay 11-7085 inhibitor also significantly inhibited NF-κB activity, and the inhibition was a function of the concentration of Bay 11-7085 used (Figure 2a).

It has been reported that the DNA-binding activity of NF-κB p50 can be modified by NO and p50 becomes S-nitrosylated and inhibits NF-κB activity (Matthews *et al.*, 1996; Dela Torre *et al.*, 1997; Marshall and Stamler, 2001). Thus, we examined whether DETA-NONOate treatment of PC-3 cells induces S-nitrosyla-tion of p50. PC-3 cells were grown in the absence or presence of DETANONOate (500 or 1000 μM) for 18 h and total cell lysates were prepared and immunopreci-pitation assay was performed as described in Materials and methods. Using anti-S-nitrosylated antibody, the S-nitrosylated proteins were immunoprecipitated and were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with

Table 1 DHT sensitizes LNCaP to TRAIL-mediated apoptosis

	TRAIL (ng/ml)		
DHT (nM)	*0*	*5*	*10*
0	5.1±1	12±2.1	17±3.8
10	6.6±0.9	18±1.1*	24±5.1*
20	6.9±1.1	23±6.1*	30.6±6.3**

LNCaP cells were treated or left untreated with DHT (10 or 20 nM) for 24 h and then treated with recombinant TRAIL (5 or 10 ng/ml) for 18 h. The cells were harvested and apoptosis was determined for cells with active caspase 3 staining by flow. The data show that DHT sensitizes LNCaP to TRAIL-mediated apoptosis. The data represent the mean of two independent experiments. *$P<0.04$, **$P<0.02$ compared with the cells treated with DHT alone

anti-NF-κB p50 antibody. S-nitrosylation of p50 was significantly enhanced following DETANONOate treatment (Figure 2b).

The relationship between DETANONOate-mediated inhibition of NF-κB and sensitization to TRAIL was examined. PC-3 cells were treated with various concentrations of Bay 11-7085 (1–5 μM) and TRAIL (5 and

10 ng/ml). Treatment with Bay 11-7085 significantly potentiated the sensitivity of PC-3 to TRAIL-mediated apoptosis, and the degree of apoptosis was a function of the concentration used (Figure 2c).

These findings demonstrate that DETANONOate inhibits NF-κB activity and results in the sensitization of PC-3 to TRAIL-induced apoptosis. Further, the results suggest that DETANONOate-mediated sensitization is via inactivation of NF-κB.

DETANONOate-mediated downregulation of Bcl-xL expression and sensitization to TRAIL

DETANONOate selectively inhibited Bcl-xL expression in PC-3 with little effect on other pro- and antiapoptotic gene products examined (Figure 3a). TRAIL has no effect on any of the gene products examined. It has been reported that Bcl-xL transcription is regulated in part by NF-κB (Mori *et al.*, 2001; Sevilla *et al.*, 2001). Thus, it was possible that DETANONOate-mediated inhibition of NF-κB (Figure 2a) was responsible for the observed DETANONOate-mediated inhibition of Bcl-xL expression (Figure 3a). This was confirmed by demonstrating that treatment of PC-3 with the NF-κB inhibitor Bay 11-7085, like DETANONOate, also inhibited Bcl-xL expression (Figure 3b). Therefore, it was possible that sensitization of PC-3 by DETANONOate to TRAIL-induced apoptosis was due in part to downregulation of Bcl-xL expression via inhibition of NF-κB. Accordingly, inhibition of Bcl-xL expression should sensitize PC-3, like NO, to TRAIL-induced apoptosis. Treatment of PC-3 with the Bcl-xL inhibitor 2-methoxyantimycin A₃ (2MAM-A3) (Tzung *et al.*, 2001) resulted in significant sensitization of the cells to TRAIL-induced apoptosis. The potentiation was a function of the concentration of 2MAM-A3 used (Figure 3c). These findings suggest that Bcl-xL is the dominant resistant factor in PC-3 cells for TRAIL-induced apoptosis, and Bcl-xL inhibition by DETANONOate via NF-κB
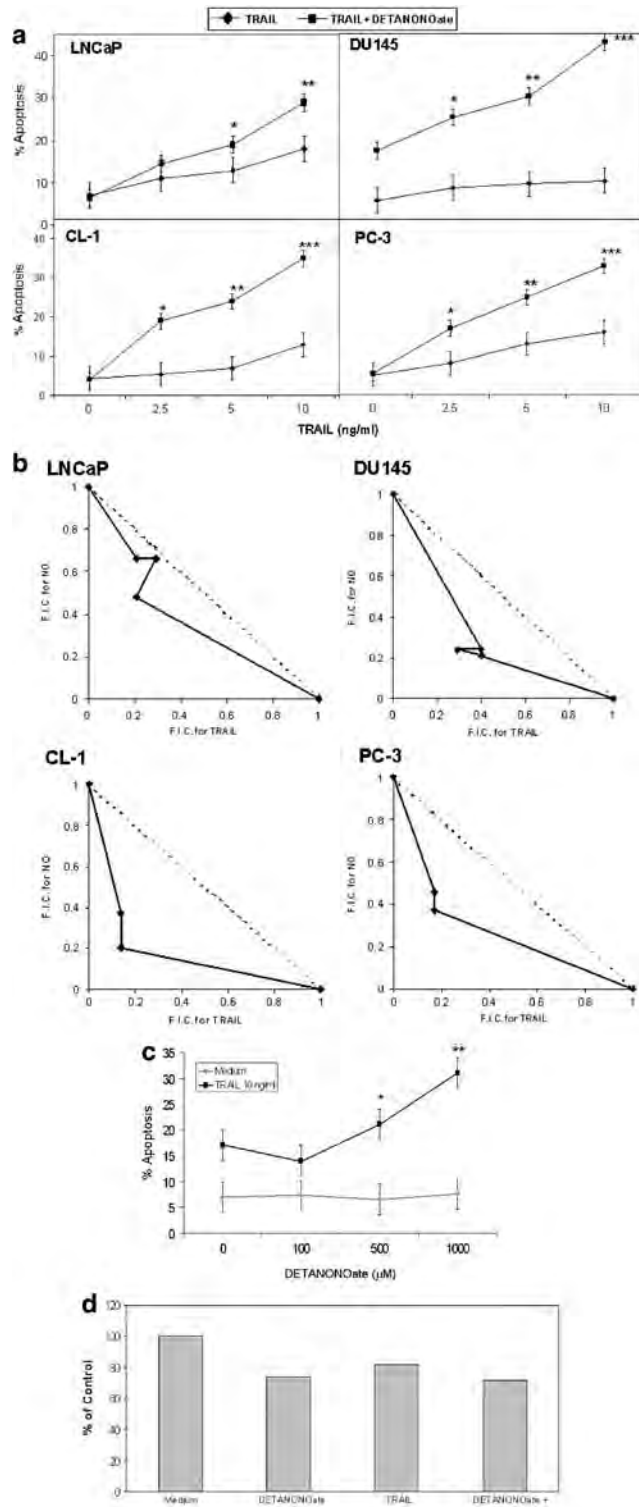
Figure 1 DETANONOate sensitizes CaP cell lines to TRAIL-mediated apoptosis. (a) The CaP cell lines DU145, CL-1, and PC-3 were grown in FBS-free medium and LNCaP cells were grown in a medium with 1% FBS. The cell lines were treated with different concentrations of TRAIL (0, 2.5, and 5 ng/ml) in the presence or absence of DETANONOate (1000 μM) for 18 h at 37° in a 5% CO₂ incubator. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analysed by flow cytometry as described in Materials and methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. *P<0.05, **P<0.02, ***P<0.004. (b) This figure establishes synergy as determined by isobologram analysis. (c) PC-3 cells were grown in FBS-free medium and were treated with TRAIL (5 ng/ml) in the presence or absence of different concentrations of DETANONOate (100, 500, and 1000 μM) for 18 h and analysed for apoptosis. Significant sensitization was observed at DETANONOate concentrations of 500 and 1000 μM. (d) The PC-3 cells were treated with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), and the combination, and viable cell recovery was examined microscopically by Trypan blue dye exclusion at 24 h. The data show that all agents inhibited cell proliferation

inactivation may be responsible for sensitization to TRAIL.

It has been reported that NF-κB activity plays an important role in the transcriptional regulation of Bcl-xL (Mori *et al.*, 2001; Sevilla *et al.*, 2001). To determine whether NF-κB activity is required for Bcl-xL transcription and to determine how DETANONOate induces selective inhibition of Bcl-xL via NF-κB, transient transfection assays were performed. PC-3 cells were transfected with the Bcl-x WT promoter and Bcl-x κB promoter reporter plasmids. At 24 h after transfection, the cells were treated with either Bay 11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or optimal

concentrations of TNF-α (50 or 100 U/ml) for 18 h. Both DETANONOate treatment and Bay 11-7085 treatment induced significant inhibition of Bcl-xL transcription, and the extent of inhibition was concentration dependent. In contrast, activation of NF-κB by TNF-α treatment induced a significant increase in Bcl-xL transcription (Figure 4). The basal luciferase activity was significantly reduced in the mutant (5 ×) compared to wild type, suggesting that Bcl-xL transcription in PC-3 is primarily regulated by NF-κB. In contrast to the findings in the wild type, the different treatments did not affect the cells transfected with the Bcl-x κB promoter (Figure 4). These results indicate that Bcl-xL transcription in PC-3 is in large part regulated by NF-κB, and inhibition of NF-κB by DETANONOate is responsible for DETANONOate-mediated downregulation of Bcl-xL expression.

Mechanism of DETANONOate-mediated sensitization to TRAIL apoptosis

We investigated the mechanism by which DETA-NONOate signals the cells leading to sensitization to TRAIL-mediated apoptosis. The effect of DETA-NONOate on the mitochondria was examined. DETA-NONOate significantly induced membrane depolarization of the mitochondria in PC-3 cells. In addition, TRAIL also significantly induced membrane depolarization, and the combination resulted in membrane depolarization that was equivalent to either DETA-NONOate or TRAIL used alone (Figure 5a). The effect of DETANONOate and TRAIL on the release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI) from the mitochondria was also examined. Both DETANONOate and TRAIL induced the release of both cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol, and the combination of DETANONOate and TRAIL resulted in more significant release of cytochrome *c*
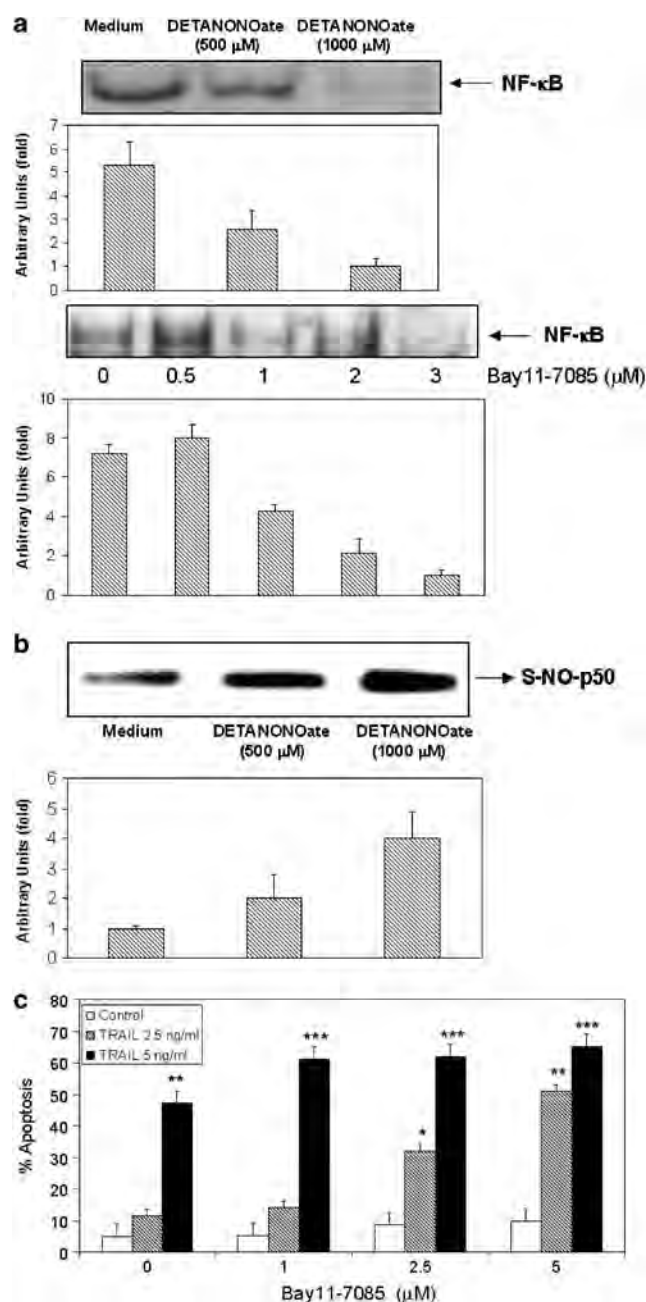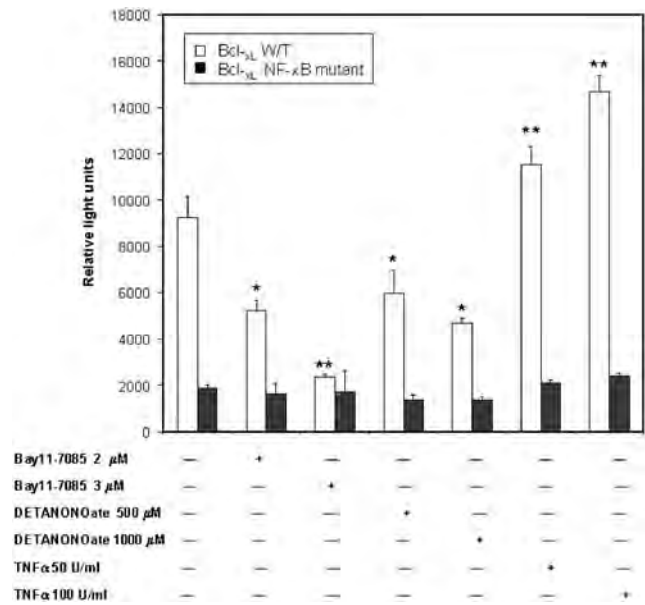
Figure 2 NF-κB is involved in TRAIL-mediated apoptosis in PC-3 cells. (**a**) Inhibition of NF-κB activity. Nuclear extracts from PC-3 cells grown in FBS-free medium were treated or left untreated with DETANONOate (500 or 1000 μM) (top panel), or treated with different concentrations of the specific NF-κB inhibitor Bay 11-7085 (0, 0.5, 1, 2, and 3 μM) (bottom panel), and were analysed by EMSA to assess NF-κB DNA-binding activity. Relative NF-κB binding activity was determined by densitometry analysis. The findings demonstrate that treatment of PC-3 cells with DETA-NONOate results in inhibition of NF-κB activity. (**b**) Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50) upon DETANONOate (500 and 1000 μM, 18 h) treatment. Total cell lysates were used in an immunoprecipitation assay using protein A beads as described in Materials and methods. S-nitrosylated proteins were precipitated and the membranes were immunoblotted with anti-NF-κB p50 polyclonal antibody. The results demonstrate that p50 was S-nitrosylated. The findings are representative of two independent experiments. (**c**) Sensitization of PC-3 to TRAIL apoptosis by inhibition of NF-κB. PC-3 cells were treated with TRAIL (2.5 and 5.0 ng/ml) in the presence or absence of various concentrations of Bay11-7085 and apoptosis was assessed. The findings demonstrated that Bay11-7085 sensitizes PC-3 cells to TRAIL-mediated apoptosis. *$P < 0.05$, **$P < 0.02$, ***$P < 0.002$

a

b

c
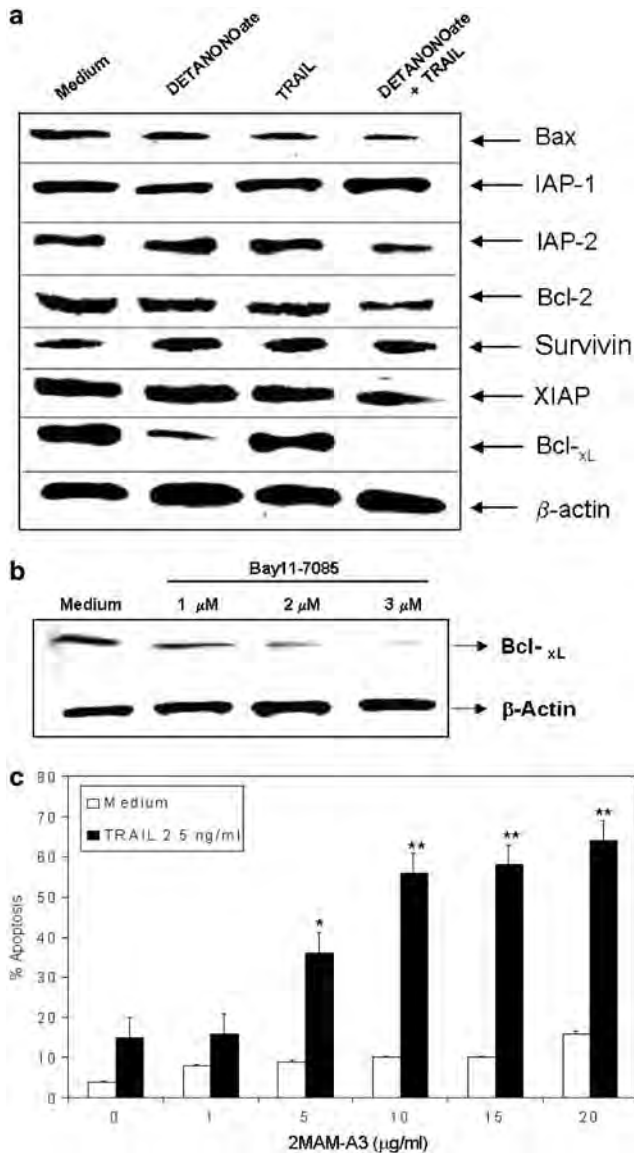
Figure 3 DETANONOate-mediated downregulation of Bcl-$_{xL}$ expression and sensitization to TRAIL-mediated apoptosis. (**a**) PC-3 cells were grown in serum-free medium and the cells were treated or not treated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted and separated by SDS–PAGE and transferred onto nitrocellulose membranes as described in Materials and methods. DETANONOate selectively downregulated Bcl-$_{xL}$ expression. Treatment of PC-3 with different concentrations of the NF-κB inhibitor Bay11-7085 resulted in inhibition of Bcl-$_{xL}$ expression. (**b**) PC-3 cells were treated with different concentrations of the Bcl-$_{xL}$ inhibitor 2MAM-A3 for 5 h and then treated with TRAIL (2.5 ng/ml) for 18 h and analysed for apoptosis. The data show that 2MAM-A3 sensitizes PC-3 to TRAIL apoptosis. *$P = 0.036$, **$P < 0.02$

Figure 4 Inhibition of Bcl-xL transcription by DETANONOate. A Bcl-$_{xL}$ promoter fragment spanning −640 to −9 relative to the transcriptional start site (Bcl-$_{xL}$ WT promoter) and another fragment missing the NF-κB binding sequence (Bcl-$_{xL}$ ΔκB promoter) were cloned into the pGL2-Basic luciferase reporter vector (Lee *et al.*, 1999). PC-3 cells were transfected with 20 μg of the indicated reporter plasmid and then treated with the specific NF-κB inhibitor Bay11-7085 (2 or 3 μM), DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml). The samples were harvested 18 h after treatment and assessed for luciferase activity. The data show that DETANONOate inhibits Bcl-$_{xL}$ transcription by inhibition of luciferase activity. The data are representative of two experiments. *$P = 0.031$, **$P < 0.02$

of the mitochondria by both TRAIL and DETA-NONOate used in combination resulted in complementation and type II mitochondria-mediated sensitization of the cells to TRAIL-mediated apoptosis.

Discussion

This study presents evidence that the NO donor, DETANONOate, sensitizes androgen-dependent and -independent CaP cell lines to TRAIL-mediated apoptosis via inhibition of NF-κB activity and downregulation of Bcl-$_{xL}$ expression. The inactivation of NF-κB by DETANONOate was via S-nitrosylation of NF-κB p50. The role of NF-κB in the transcriptional activity of Bcl-$_{xL}$ expression was demonstrated by the use of NF-κB inhibitors and by the use of a luciferase reporter construct driving the Bcl-$_{xL}$ promoter. Treatment with DETANONOate or Bay11-7085 inhibited significantly luciferase activity whereas TNF-α augmented the basal activity. In contrast, removal of the putative NF-κB-binding sequence from the promoter resulted in low constitutive level of luciferase activity and this basal level was not affected by DETANONO-ate or by the NF-κB inhibitor. Inhibition of either NF-κB or Bcl-$_{xL}$ by chemical inhibitors sensitized significantly to TRAIL-mediated apoptosis. The synergy achieved in apoptosis by combination treatment was the

and Smac/DIABLO (Figure 5b). In addition, there was little activation of procaspase 8 and procaspase 9 by either DETANONOate or TRAIL used alone, although the combination resulted in significant activation of procaspase 8 and procaspase 9 (Figure 5c). These findings demonstrate that DETANONOate selectively inhibits Bcl-$_{xL}$ expression (Figure 3a), and the activation

result of complementation in the activation of the type II mitochondrial pathway for apoptosis. Thus, both TRAIL and DETANONOate partially activate the mitochondria, with membrane potential depolarization and some release of cytochrome *c* and Smac/DIABLO, although each alone could not activate caspase 9. The combination of DETANONOate and TRAIL, however, resulted in caspase 9 and 3 activation and apoptosis. Altogether, these findings provide a novel mechanism of Bcl-xL regulation by NO via NF-κB inhibition and suggest that NO donors may be of potential therapeutic value as sensitizing agents when used in combination with TRAIL in the treatment of TRAIL-resistant tumor cells.

Our findings demonstrate that DETANONOate sensitized both androgen-dependent (LNCaP) and androgen-independent (DU145, PC-3, and CL-1) CaP

cells to TRAIL-induced apoptosis and synergy was achieved. Previous findings from our laboratory have demonstrated that subtoxic concentrations of chemotherapeutic drugs like actinomycin D sensitized the above CaP tumor cells to TRAIL apoptosis (Zisman *et al.*, 2001). Actinomycin D was shown to downregulate X-linked inhibitor of apoptosis (XIAP) selectively and, thus, facilitated the TRAIL-induced apoptotic pathway (Ng *et al.*, 2002). The role of XIAP in resistance was corroborated in experiments showing that transfection with Smac/DIABLO, which inhibits inhibitor of apoptosis proteins (IAPs), sensitizes cells to TRAIL apoptosis in the absence of actinomycin D (Ng and Bonavida, 2002b). The present findings with DETANONOate, however, are different such that NO selectively inhibits NF-κB and Bcl-xL expression in the absence of modification of XIAP expression and sensitizes the cells to TRAIL apoptosis. These findings demonstrate that the regulation of apoptosis by TRAIL in the CaP cell lines studied may be influenced by various antiapoptotic members of the signaling pathway and the inhibition of one such member, such as XIAP or Bcl-xL, was sufficient to reverse the resistance to TRAIL.

In CaP, NF-κB contributes to the progression to androgen independence and increases invasive and metastatic properties (Palayoor *et al.*, 1999; Rayet and Gelinas, 1999). Basal levels of NF-κB are detected in normal prostatic epithelial cells and the androgen-dependent CaP cell line LNCaP (Palayoor *et al.*, 1999; Huang *et al.*, 2001). It has been reported that crosstalk occurs between NF-κB signaling and steroid receptor signaling pathways (Palvimo *et al.*, 1996; McKay and Cidlowski, 2000). We show that treatment of LNCaP
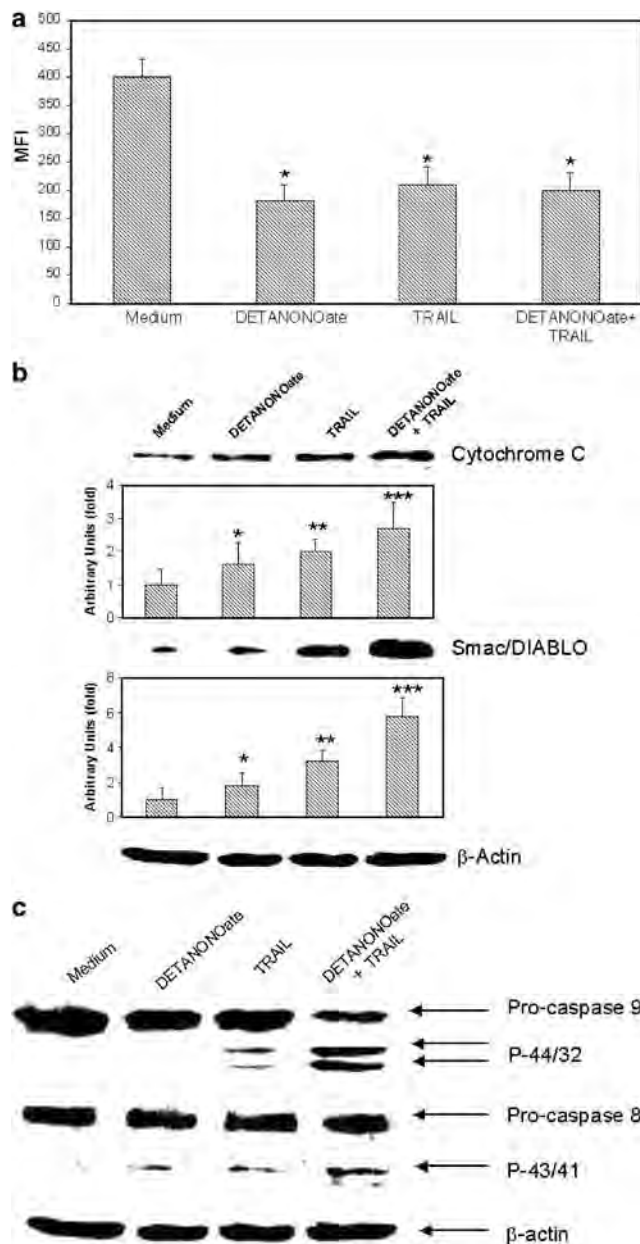
Figure 5 Mitochondrial membrane depolarization, release of cytochrome *c* and Smac/DIABLO into the cytosol, and activation of caspases 8 and 9. (**a**) Mitochondrial membrane activation. PC-3 cells were grown in FBS-free medium and treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. The PC-3 cells were then stained with DiOC6 and then analysed by flow cytometry. The findings demonstrate that DETANONOate, TRAIL, and the combination induce significant mitochondrial depolarization. The data represent the mean fluorescence intensity (MFI), and are the mean of three independent experiments. *$P < 0.05$, medium *vs* cells treated. (**b**) Release of cytochrome *c* and Smac/DIABLO. PC-3 cells were grown in FBS-free medium and were treated or left untreated for 18 h with DETANONOate (1000 μM), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted from the culture. The purified fraction of cytosolic protein was separated by SDS–PAGE and transferred onto the nitrocellulose membrane as described in Materials and methods. The membrane was stained with polyclonal anti-human cytochrome *c* antibody (top panel) or anti-Smac/DIABLO antibody (bottom panel). The blots represent one of two separate experiments. The data show that DETANONOate and TRAIL induce some release of both cytochrome and Smac/DIABLO, and the combination releases higher levels. The relative cytochrome *c* and Smac/DIABLO expression was determined by densitometric analysis of the blot. *$P < 0.05$, **$P < 0.03$, ***$P < 0.002$ medium *vs* cells treated. (**c**) Activation of caspases 8 and 9. PC-3 cells were treated as described above. The activation of caspases 8 and 9 was determined by Western blot. There was some activation of caspase 8 by DETANONOate and some activation of caspase 9 by TRAIL. However, the combination resulted in significant activation of both caspases
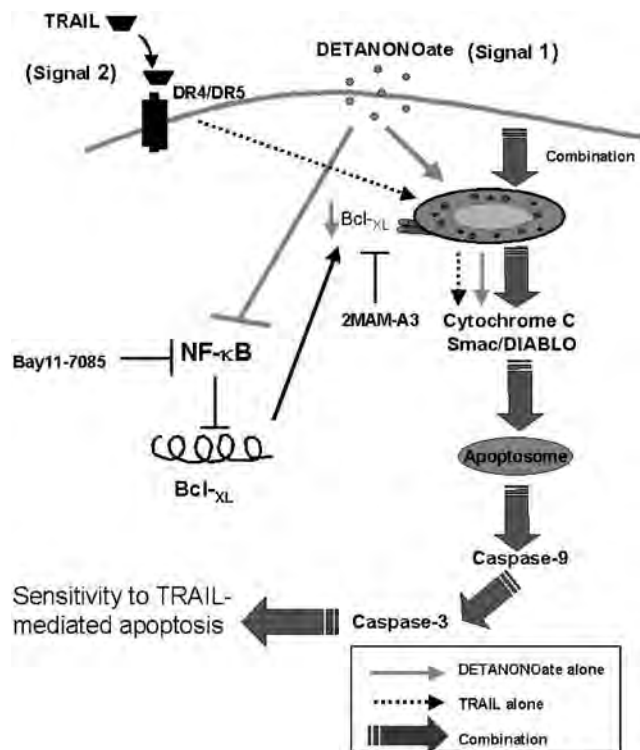
Figure 6 Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL. This figure schematically demonstrates that treatment of PC-3 cells with DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by DETANONOate, which partially inhibits NF-κB activity, and this leads to downregulation of Bcl-xL transcription. In addition, DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome c and Smac/DIABLO into the cytosol in the absence of downstream activation of caspase 9. Signal 2 is provided by TRAIL, which also partially activates the mitochondria with some release of cytochrome c and Smac/DIABLO in the absence of caspase 9 activation. However, the combination treatment results in significant activation of the mitochondria and release of high levels of cytochrome c and Smac/DIABLO, activation of caspases 9 and 3, resulting in apoptosis. The two-signal model is corroborated by the use of specific inhibitors in which inhibition of NF-κB by Bay11-7085 was sufficient to sensitize the CaP cells to TRAIL-induced apoptosis concomitant with downregulation of Bcl-xL expression. The role of Bcl-xL in the regulation of TRAIL apoptosis was corroborated by the use of the chemical inhibitor 2MAM-A3, which also sensitized the cells to apoptosis

with DHT sensitized the cells to TRAIL via inhibition of NF-κB. In contrast, androgen-independent CaP cells PC-3 and DU-145 have elevated NF-κB activity and this was confirmed here (data not shown). In addition, PC-3 and DU-145 cells have constitutively active IκB kinase complex (IKK), which activates NF-κB (Gasparian et al., 2002). Thus, constitutive activation of NF-κB plays a central role in the resistance to CaP cell line to therapeutic agents.

The present findings demonstrate that DETANONOate inhibits NF-κB activity. It has been shown that high levels of NO inhibit NF-κB activity by several mechanisms. For instance, DETANONOate inhibits the phosphorylation and subsequent degradation of IκB-α, which prevents nuclear localization of NF-κB (Katsuyama et al., 1998). Also, NO may quench reactive oxygen species that are responsible for the activation of NF-κB (Garban and Bonavida, 2001b). In addition, recent studies demonstrate that NO induces S-nitrosylation of NF-κB p50 and reduces its DNA-binding activity (Connely et al., 2001; Marshall and Stamler, 2001). NF-κB displays redox-sensitive DNA-binding activity (Chinenov et al., 1998; Tell et al., 1998). This redox sensitivity is conferred by a single cysteine residue within the DNA-binding site (Matthews et al., 1993; Marshall and Stamler, 2001). In this study, we demonstrate that NF-κB binding activity was significantly decreased after treatment with DETANONOate (Figure 2a). We also demonstrate that DETANONOate induced strongly S-nitrosylation of NF-κB p50 (Figure 2b) in agreement with the findings of Marshall and Stamler (2001) and Connely et al. (2001).

Recent studies demonstrated that Bcl-2 and Bcl-xL block apoptosis induced by physiological agents such as TRAIL in PC-3, DU-145, and LNCaP CaP cells (Rokhlin et al., 2001). In addition, overexpression of Bcl-xL in LNCaP and PC-3 cells desensitized the cells to the effects of cytotoxic chemotherapeutic agents (Li et al., 2001). However, downregulated endogenous levels of Bcl-xL, but not Bcl-2, induced a marked increase in chemosensitivity (Lebedeva and Stain, 2000). These results suggest the important role of Bcl-xL in the resistance to apoptosis induced by cytotoxic agents like TRAIL in CaP. It is noteworthy that our results demonstrate that DETANONOate treatment induces selective downregulation of Bcl-xL expression and sensitizes the CaP cells to TRAIL-induced apoptosis. Further, inhibition of Bcl-xL function by 2MAM-A3 sensitizes the cells to TRAIL apoptosis. These findings corroborate the role of Bcl-xL in the regulation of resistance of CaP to chemotherapy and TRAIL.

The mechanism by which NO induces inhibition of Bcl-xL expression was examined. Previous findings demonstrated that the Bcl-xL promoter contains an element that binds NF-κB transcription factors and supports transcriptional activation by members of this family (Lee et al., 1999). It was possible that DETANONOate inhibits NF-κB and this, in turn, inhibits Bcl-xL transcription. We demonstrate here that DETANONOate inhibits Bcl-xL expression via inactivation of NF-κB activity. This was shown by using a luciferase reporter construct driving the Bcl-xL promoter. Treatment with DETANONOate or Bay 11-7085 (which selectively and irreversibly inhibits the induced phosphorylation of IκB without affecting the constitutive IκB-α phosphorylation; Pierce et al., 1997) significantly inhibited the high constitutive luciferase activity. However, there was little luciferase activity following the removal of the putative NF-κB-binding sequence from the promoter and neither DETANONOate nor Bay 11-7085 had any effect. These results directly demonstrate that Bcl-xL expression in PC-3 is primarily regulated by

NF-κB and inhibition of NF-κB, in turn, inhibits Bcl-xL transcription.

NO, synthesized from L-arginine by NO synthase, is a small, diffusible, highly reactive molecule with dual regulatory roles under physiological and pathological conditions (Schmidt and Walter, 1994). NO can promote apoptosis (proapoptosis) in some cells, whereas it inhibits apoptosis (antiapoptosis) in other cells. This dichotomy depends on the rate of NO production and the interaction with biological molecules such as iron, thiol, proteins, and reactive oxygen species (Schmidt, 1992; Stamler, 1994). High concentrations of NO and also long-lasting production of NO such as by DETANONOate used here act as proapoptotic modulators (Messmer and Brune, 1996; Poderoso et al., 1996; Jun et al., 1999; So et al., 1998; Di Nardo et al., 2000). The present findings are consistent with the proapoptotic effects of the high levels of NO used to sensitize CaP cells.

NO binds to cytochrome c oxidase (complex IV) in the mitochondrial electron transfer chain (Poderoso et al., 1996). Under this condition, superoxide generated from mitochondria interacts with NO to form peroxynitrite, which induces mitochondrial dysfunction and cytochrome c release. NO also generates ceramide, which induces cytochrome c release from mitochondria (Ghafourifar et al., 1999). Our results clearly show that DETANONOate induces activation of the mitochondria pathway, including mitochondrial membrane depolarization (Figure 3a) and some release of both cytochrome c and Smac/DIABLO (Figure 3b). The participation of the mitochondria is not complete because we demonstrate that downstream caspases are not activated. Caspase activation, however, resulted from the combination of DETNONOate and TRAIL. Recent studies have shown that caspase 8 activation is necessary but not sufficient for TRAIL-mediated apoptosis in prostate carcinoma cells (Rokhlin et al., 2002), suggesting the important participation of the mitochondria-dependent pathway in TRAIL-mediated apoptosis. Further, our findings with DETANONOate are consistent with those of Lee et al. (2001), who reported that sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway.

This study demonstrates that the combination of NO donor and TRAIL can sensitize TRAIL-resistant CaP to TRAIL-induced apoptosis. This combination treatment is a result of two complementary signals induced by each agent alone (Ng and Bonavida, 2002a; schematically diagrammed in Figure 6). Signal 1 results from NO-induced perturbation of the mitochondria, inhibition of NF-κB activity, and downregulation of Bcl-xL expression. Signal 1 alone is not sufficient to promote the cells toward apoptosis. Signal 2 is induced by TRAIL, which activates the mitochondria slightly, but not sufficient to activate the apoptosome and induce apoptosis. However, combination of the two signals results in complementation and activation of the mitochondrial pathway and activation downstream of caspases 9 and 3 resulting in apoptosis. Thus, the

findings of this report reveal that NO can selectively inhibit the expression of the antiapoptotic resistant factor Bcl-xL via inhibition of NF-κB activity. The findings also reveal new targets for intervention affecting NF-κB activity or Bcl-xL expression and whose modification may revert resistance of CaP to TRAIL apoptosis. Thus, NO donors or Bcl-xL inhibitors may be useful in the treatment of TRAIL-resistant tumors in combination with TRAIL or TRAIL agonists such as antibody against DR4/DR5 (DR: death receptor) (Ichikawa et al., 2001).

Materials and methods

Reagents

The anti-Bcl-xL and anti-β-actin monoclonal antibodies were purchased from Santa Cruz (California, USA) and from Calbiochem (San Francisco, CA, USA), respectively. mAb anti-Bcl-2 was obtained from DAKO Corporation (Carpinteria, CA, USA). The polyclonal antibodies anti-XIAP, anti-IAP-1, anti-IAP-2, anticaspase 8, anticaspase 9, and survivin were obtained from Cell Signaling (San Diego, CA, USA), anti-cytochrome c from Pharmingen (San Diego, CA, USA), and anti-Smac/DIABLO from Alexis (San Diego, CA, USA). The human recombinant TRAIL and TNF-α were obtained from PeproTech Inc. (Rocky Hills, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-active caspase 3 and FITC-conjugated IgG were purchased from Pharmingen (San Diego, CA, USA). The NF-κB inhibitor Bay 11-7085 (specific inhibitor of IκBα phosphorylation; Pierce et al., 1997) was obtained from Calbiochem (San Francisco, CA, USA), and the Bcl-xL inhibitor 2MAM-A3 (binds to the hydrophobic groove of Bcl-2 and Bcl-xL) (Tzung et al., 2001) was obtained from Biomol (Plymouth, PA, USA). The DETANONOate was obtained from Alexis (San Diego, CA, USA).

Cells and culture conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent LNCaP and the androgen-independent (Tso et al., 2000) CL-1 (LNCaP-derived) cell lines were kindly provided by Dr Arie Belldegrun at UCLA. Cells were maintained as a monolayer in 80 mm² plates in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids. FBS (Life Technologies) was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/l R1881 methyltrienolone (New Life Science Products, Boston, MA, USA). The cell cultures were maintained as monolayers on plastic dishes and were incubated at 37°C and 5% carbon dioxide in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated FBS (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to treatments.

Cell treatments

Log-phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6×10^4 cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1, and PC-3 cells were synchronized by treatment with 1% FBS for 18 h prior to each experiment. The treatment of NCaP cells was in a medium with 1% of serum and the treatments of DU145, CL1, and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate, or the combination for 18 h. For the experiments of sensitization to TRAIL-mediated apoptosis by the NF-κB inhibitor Bay 11-7085, the cells were treated with different concentrations of Bay 11-7085 for 1 h and then treated with various concentrations of TRAIL for 18 h. For sensitization to TRAIL-mediated apoptosis by the Bcl-xL inhibitor 2MAM-A3, the cells were treated with different concentrations of 2MAM-A3 for 4 h, and then treated with TRAIL for 18 h.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice-cold $1 \times$ phosphate-buffered saline (PBS) and were resuspended in $100 \mu l$ of the cytofix/cytoperm solution (Pharmingen, San Diego, CA, USA) for 20 min. Thereafter, the samples were washed twice with ice-cold $1 \times$ perm/wash buffer solution (Pharmingen) and were stained with FITC-labeled anti-active caspase 3 mAb for 30 min (light protected). The samples were subsequently washed once with $1 \times$ perm/wash buffer solution and $250 \mu l$ of $1 \times$ PBS was added prior to flow cytometry analysis on a flow cytometer EPICS^R XL-MCL (Coulter, Co. Miami, FL, USA), with the System II™ Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Immunoprecipitation of S-nitrosylated NF-κB p50 (S-NO-p50)

The S-nitrosylation of NF-κB p50 was analysed by immuno-precipitation assay. The cells were grown in the presence and absence of DETANONOate (0, 500, and 1000 μM) and then harvested and pelleted at 14 000 g for 2 min. The resulting cell pellets were resuspended and dissolved in 500 μl ice-cold components of radioimmunoprecipitation assay (RIPA) buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2 μg of rabbit anti-S-nitrosylated proteins polyclonal Ab (Calbiochem, San Diego, CA, USA) and were subsequently incubated with 30 μl Immuno-Pure Plus Immobilized protein A (Lindmark *et al.*, 1983) (Pierce, Rockford, IL, USA) for 4 h at 4°C on a shaking platform. The lysates were centrifuged for 1 min at 14 000 g and the supernatants were discarded. The immunoprecipitates were washed twice with 1.0 ml of ice-cold RIPA buffer prior to assay. The immunoprecipitates were resolved on a 12% SDS–PAGE gel and subsequently immunoblotted with anti-NF-κB p50 polyclonal Ab (1 : 2000 dilution) (Active Motif, Carlsbad, CA, USA). The immunostaining was visualized by autoradiography.

Luciferase Bcl-xL promoter reporter assay

The Bcl-xL WT promoter luciferase (Bcl-x WT promoter) reporter plasmid and the Bcl-xL promoter missing the NF-κB-binding sequence (Bcl-x κB promoter) have been previously characterized (Lee *et al.*, 1999). PC-3 cells were transfected by electroporation using pulses at 250 V/975 μF (Bio-Rad), with 20 μg of Bcl-x WT promoter or Bcl-x κB promoter. After transfection, the cells were allowed to recover overnight and were cultured in six-well plates. Cells were treated with the specific NF-κB inhibitor Bay 11-7085 (2 or 3 μM), NO donor DETANONOate (500 or 1000 μM), or TNF-α (50 or 100 U/ml) for 18 h. Cells were then harvested in $1 \times$ lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate.

Measurement of mitochondrial membrane depolarization

The mitochondria-specific dye 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (Molecular Probes Inc., Eugene, OR, USA) was used to measure the mitochondrial potential. PC-3 cells were grown in six-well plates and were treated with TRAIL (2.5 ng/ml) and/or DETANONOate (1000 μM) simultaneously. After treatments, the cells were collected at 18 h. A total of 50 μl of 40 μM ($DiOC_6$) was loaded to stain the cells for 30 min immediately after the cells were collected. The cells were detached by using PBS supplemented with 0.5 μM ethylene-diaminetetraacetic acid (EDTA), washed twice in PBS, resuspended in 1 ml of PBS, and analysed by flow cytometry as reported (Ng *et al.*, 2002).

Western blot analysis

PC-3 cells were cultured at a low FBS concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with TRAIL (2.5 ng/ml), DETANONOate (1000 μM), or the combination. The cells were then lysed at 4°C in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN, USA). Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). An aliquot of total protein lysate was diluted in an equal volume of $2 \times$ SDS sample buffer, 6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 10 min. The cell lysates (40 μg) were then electrophoresed on 12% SDS–PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi *et al.*, 2001). Levels of β-actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) using the public domain NIH Image J Program (also available via the internet).

Isolation of cytosolic fraction and determination of cytochrome c and Smac/DIABLO content

PC-3 cells were grown under the conditions explained for Western blot. At the end of the incubation period, the cells were recovered with $1 \times$ PBS/EDTA, washed with $1.0 \times$ PBS/0.1% BSA and resuspended in two volumes of homogenization buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM 1,4-dithiothreitol (DTT), one tablet of Complete Mini protease inhibitor cocktail in 250 mM sucrose medium). After 30 min on ice, the cells were disrupted by 40 strokes of a dounce glass homogenizer using a loose pestle (Bellco Glass Inc., Vineland, NJ, USA). The homogenate was centrifuged at 2500 g at 4°C

for 5 min to remove nuclei and unbroken cells. The mitochondria were pelleted by spinning the homogenate at $16\,000\,g$ at 4°C for 30 min. The supernatant was removed and filtered through 0.1 μm Ultrafree MC filters (Millipore) to obtain the cytosolic fraction and was spun down at $16\,000\,g$ at 4°C for 15 min. The protein concentration of the supernatant was determined by the DC assay kit and was mixed with $2\times$ Laemmli sample buffer and analysed by SDS–PAGE for determination of cytochrome c and Smac/DIABLO contents in the cytosolic fraction as previously reported (Jazirehi et al., 2003).

Nuclear extracts preparation

Nuclear extract preparations were carried out as previously described by our laboratory (Garban and Bonavida, 2001b). Briefly, cells (10^{6}) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA, USA). After washing, cells were lysed in 1 ml of NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl$_2$, and 0.5% NP-40) on ice for 5 min. Samples were centrifuged at $300\,g$ at 4°C for 5 min. The pellet was washed twice in NP-40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN, USA). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at -80°C.

EMSA

Nuclear proteins (5 μg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB using EMSA Kit Panomics™ (Panomics Inc., Redwood City, CA, USA) following the manufacturer's instructions (Vega et al., 2004). A measure of 10 μl was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide sequences for NF-κB are as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Harada et al., 1994). Relative concentrations were assessed by densitometric analysis as mentioned above.

Isobologram analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/DETONONOate combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5, and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 μM) as described (Berenbaum, 1978). Combinations yielding a cytotoxicity of $30\pm5\%$ were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose–response curves using active caspase 3 analysis for LNCaP, DU145, CL-1, and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18 h.

Statistical analysis

The experimental values were expressed as the mean $\pm$ s.d. for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's t-test was used for comparison between two groups. Significant differences were considered for probabilities $<5\%$ ($P<0.05$).

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Raf-1 Kinase Inhibitor Protein: Structure, Function, Regulation of Cell Signaling, and Pivotal Role in Apoptosis

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The acquisition of resistance to conventional therapies such as radiation and chemotherapeutic drugs remains the major obstacle in the successful treatment of cancer patients. Tumor cells acquire resistance to apoptotic stimuli and it has been demonstrated that conventional therapies exert their cytotoxic activities primarily by inducing apoptosis in the cells. Resistance to radiation and chemotherapeutic drugs has led to the development of immunotherapy and gene therapy approaches with the intent of overcoming resistance to drugs and radiation as well as enhancing the specificity to eliminate tumor cells. However, cytotoxic lymphocytes primarily kill by apoptosis and, therefore, drug-resistant tumor cells may also be cross-resistant to immunotherapy. To evade apoptosis, tumor cells have adopted various mechanisms that interfere with the apoptotic signaling pathways and promote constitutive activation of cellular proliferation and survival pathways. Thus, modifications of the antiapoptotic genes in cancer cells are warranted for the effectiveness of conventional therapies as well as novel

immunotherapeutic approaches. Such modifications will avert the resistant phenotype of the tumor cells and will render them susceptible to apoptosis. Current studies, both *in vitro* and preclinically *in vivo*, have been aimed at the modification and regulation of expression of apoptosis-related gene products and their activities. A novel protein designated Raf-1 kinase inhibitor protein (RKIP) has been partially characterized. RKIP is a member of the phosphatidylethanolamine-binding protein family. RKIP has been shown to disrupt the Raf-1–MEK1/2 [mitogen-activated protein kinase–ERK (extracellular signal-regulated kinase) kinase-1/2]–ERK1/2 and NF-κB signaling pathways, via physical interaction with Raf-1–MEK1/2 and NF-κB-inducing kinase or transforming growth factor β-activated kinase-1, respectively, thereby abrogating the survival and antiapoptotic properties of these signaling pathways. In addition, RKIP has been shown to act as a signal modifier that enhances receptor signaling by inhibiting G protein-coupled receptor kinase-2. By regulating cell signaling, growth, and survival through its expression and activity, RKIP is considered to play a pivotal role in cancer, regulating apoptosis induced by drugs or immune-mediated stimuli. Overexpression of RKIP sensitizes tumor cells to chemotherapeutic drug-induced apoptosis. Also, induction of RKIP by drugs or anti-receptor antibodies sensitizes cancer cells to drug-induced apoptosis. In this review, we discuss the discovery, structure, function, and significance of RKIP in cancer. © 2004 Elsevier Inc.

I. INTRODUCTION: THE PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN FAMILY

Raf-1 kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine-binding protein (PEBP) family. This family is a highly conserved group of proteins found in a variety of organisms from plants to *Drosophila* to mammals. Analysis of databases for proteins having homology to PEBP has revealed no significant sequence similarity with other proteins, suggesting the unique characteristics of this family of proteins (Banfield *et al.*, 1998). PEBPs are 21- to 23-kDa (human PEBP is 187 amino acids) basic cytosolic proteins that were originally purified from bovine brain while searching for soluble cytosolic proteins with the property of binding hydrophobic ligands (Schoentgen and Jolles, 1995). New forms of mammalian PEBPs were found from cDNA libraries from mice and rats while searching for novel cDNAs with a role in spermiogenesis (Banfield *et al.*, 1998; Hickox *et al.*, 2002; Simister *et al.*, 2002) (Table I and Fig. 1). Binding studies have shown that PEBPs have an affinity for phosphatidylethanolamine; nucleotides such as GTP, GDP, and small GTP-binding proteins; as well as for other hydrophobic ligands (Schoentgen and Jolles, 1995).

There are 13 identified mammalian PEBP sequences with a highly conserved central region (residues 60–126) believed to be essential for PEBP function and binding to G proteins. The PEBPs can be grouped into four subfamilies based on their sequences: PEBP-1, -2, -3, and -4 (Fig. 1) (Banfield *et al.*, 1998; Simister *et al.*, 2002). No PEBP-2 homologs have been

Table I Properties of Phosphatidylethanolamine-Binding Protein Family Members

PEBP	Description	Ref.
Expression	Family of highly conserved proteins found in plants, *Drosophila*, and mammals	Vallee *et al.* (1999)
Discovery	Initially found as the precursor of hippocampal cholinergic neurostimulatory peptide (HCNP)	Maki *et al.* (2002)
Subgroups	13 mammalian PEPB sequences identified. These proteins are grouped into 4 groups based on sequence homology (PEBP1–4)	Simister *et al.* (2002)
PEBP functions	1. Inhibition of serine proteases 2. Activation of G protein	Hengst *et al.* (2001) Kroslak *et al.* (2001) Yeung *et al.* (2000) Yeung *et al.* (2001)
RKIP promoter	AP-1, c-Fos, c-Jun, Sp-1, YY1, WT1, Zeste, IK-1, -2, TAF-1, Hb, GAGA factor, AP-4, CP-1, ATF	TESS master analysis[a]
Molecular mass	21–23 kDa (187 amino acids in human), located on chromosome 12	Schoentgen and Jolles (1995)
RKIP functions	1. Inhibition of the ERK1/2 pathway 2. Inhibition of the NF-κB pathway 3. Inhibition of GRK2 4. Role in apoptosis 5. Role in suppression of metastasis	Yeung *et al.* (2000) Yeung *et al.* (2001) Lorenz *et al.* (2003) Chatterjee *et al.* (2003); Fu *et al.* (2003) Jazirehi *et al.* (2004a)

[a]TESS, Transcription Element Search Software.

identified in humans. The rat and mouse PEBPs, rPEBP-2 and mPEBP-2, respectively, share 91% sequence identity. mPEBP-2 shares 84% sequence identity with human PEBP-1 (hPEBP-1). hPEBP-1 shares 79% sequence homology with the first identified murine PEBP (mPEBP-3). The first 40 residues in the NH$_2$-terminal end vary among the subfamilies, with most variation observed within the first 10 amino acids. Members of the PEBP-1, -2, and -3 subfamilies are approximately 190 residues in length. Members of the fourth subfamily, PEBP-4, have two insertions and one deletion in the protein sequence that distinguish this group from the first three. The two insertions (between residues 55–56 and 102–103 in hPEBP-1) and a single deletion (residues 131–134 in hPEBP-1) are located in loop regions identified by crystal structures and are believed not to affect the protein folding (Simister *et al.*, 2002) (Fig. 1).

A

```
1    mpvdlskswsg plslgevdeq pqtplhvtya gaavdelgkv ltptqvknrp tsiswdglds
61   gklylvtvd pdapsrkdpk yrewhhflvv nmkgndlssg tvlsdygvgg ppkgtglhry
121  vwlvyeqadrp lkcdepilsn rsgdhrgtk vasifrtkyel rapvagtcyq aewddlvpkl
181  yeqls
```

B

Sequence alignment figure (residue blocks beginning at positions 1, 103, 162, and 187).

C

Acetyl-Ala-Ala-Asp-Ile-Ser-Gln-Trp-Ala-Gly-Pro-Leu

Fig. 1 (A) Protein sequence of RKIP (Banfield *et al.*, 1998). (B) Sequence alignment of all available mammalian PEBP proteins, prepared with MULTALIN (Corpet, 1988). The proteins are divided into four families according to sequence homology. The first 33 and 40 N-terminal residues for hPEBP-4 and mPEBP-4, respectively, are not shown (Simister *et al.*, 2002). (C) Hippocampal cholinergic neurostimulating peptide (HCNP) (Seddiqi *et al.*, 1996).

Many organisms have several forms of PEBPs. For example, evaluation of protein sequence databases for the plants *Arabidopsis thaliana* and *Oryza sativa* (rice) has shown at least six sequences belonging to the PEBP family of proteins. Also, study of the genome of higher organisms such as the fruit fly (*Drosophila melanogaster*) led to the identification of at least five PEBP paralogs. A variety of ligands such as phospholipids, opioids, and odorant molecules are reported to bind to various members of this family (Banfield *et al.*, 1998).

Specific antibody against the bovine brain protein has revealed the presence of this protein in a variety of tissues such as in bovine liver, soluble extracts of rat and mouse brain, as well as human platelets (Bernier and Jolles, 1984). Expression of PEBP mRNA has been detected in all mammalian tissues tested, with high levels in spermatids, brain oligodendrocytes, Purkinje cells, and specific cortical and hippocampal neuronal cell layers. PEBP is believed to be the precursor of the hippocampal neurostimulating peptide (HCNP), an undecapeptide that is involved in the differentiation of neurons in the medial septal nucleus, enhancing the synthesis of choline acetyltransferase (Seddiqi *et al.*, 1996) (Fig. 1C).

The high affinity of PEBP for phospholipids as well as its high expression levels in growing cells might suggest a possible role in membrane organization and biogenesis (Schoentgen *et al.*, 1987). Members of the PEBP family in yeast and mouse, evolutionary distant organisms, inhibit proteolytic activity, which suggests that this function might be a common feature of the PEBPs. Amino acid analysis reveals no obvious secretion signal. Examining the binding affinities of PEBP in living organisms has revealed that PEBP directly interacts with negative membrane microdomains, with a small cavity at the protein surface serving as the binding site of the polar head of phosphatidylethanolamine. The N and C termini exposed at the protein surface appear to interact with membranes (Vallee *et al.*, 2001).

A number of regions in PEBPs are believed to be functionally important. Evaluation of the human crystal structure based on diffraction properties has revealed few important regions with important structural and functional roles (Banfield *et al.*, 1998; Simister *et al.*, 2002). These regions are close to the ligand-binding site and are required to keep the correct structure of this site to function properly. These regions include (1) the DPDxPx_nH motif (residues 69–86, where n is 11 in all mammalian proteins and the second proline adopts a *cis*-peptide conformation), (2) the GxHR motif (residues 116–119), and (3) the nonprolyl *cis*-peptide bond conformation adopted by glutamine residue 83.

The previously-described domains are participants in determining the local structure at this site. The sequence and structure of DPDxPx_nH and GxHR regions are conserved in mPEBP-2. The high electron density observed within this region as well as the localization at the apex of the region

with dipole moment suggest that this site might be the expected site for binding to the negatively charged part of the membrane (Banfield et al., 1998).

The anionic ligand-binding site in mouse PEBP-2 (mPEBP-2) has been shown to be crucial to the function of PEBPs. The ligand-binding site of mPEBP-2 has a highly conserved homology in mammalian and plant structures. Examination of the N-terminal region of the PEBP suggests that this part is cleaved from the mammalian forms to release the bioactive HCNP (Simister et al., 2002).

The mechanism of action of the PEBP subgroups is complex. The plant PEBP orthologs, Terminal flower 1, Self-pruning, and Centroradialis, are known to be involved in the regulation of flowering signaling and meristem growth (Frayne et al., 1999). In nematodes, PEBPs are found to be part of the secreted cell surface proteins and protect against host immunological responses (Frayne et al., 1999). The *Drosophila* PEBP homologs serve as putative odorant-binding effector molecules expressed in different subsets of olfactory hairs (Frayne et al., 1999).

Mammalian PEBPs have been found to regulate serine proteases by selectively inhibiting their activities. Serine proteases are involved in many processes in the nervous system, where they play important roles in development and tissue homeostasis. Serine proteases degrade components of the extracellular matrix to allow outgrowth of neuronal processes or cell migration, promote cell death, and act as mitogenic or survival factors. The activity of proteases is regulated by their inhibitors and disturbances in this regulation have been proposed to cause pathological disorders such as Alzheimer's disease (Hengst et al., 2001). Several serine proteases have been detected in the central nervous system, including tissue-type plasminogen activator (t-PA), chymotrypsin, neuropsin, elastase, and thrombin (Hengst et al., 2001). PEBP inhibits chymotrypsin, a serine protease with specificity for hydrophobic and aromatic amino acids, as well as thrombin, which cleaves only after basic amino acids. The mechanism and the active site of PEBP that lead to the inhibition of serine proteases are not known yet. Finally, PEBP inhibits neuropsin, but not the activities of trypsin, t-PA, and pancreatic elastase (Hengst et al., 2001).

Furthermore, PEBPs have been shown to associate with cellular membranes and thus could participate in G protein-dependent signaling in a membrane-dependent fashion (Banfield et al., 1998). On binding of neurotransmitters, growth factors, and hormones, the G protein-coupled receptor (GPCR) is activated. On activation, GDP is replaced by GTP on the G_α subunit and thus separation of G_α from the G_β and G_γ subunits. G_α can then activate the adenylate cyclase, leading to the generation of cAMP from ATP and thus activation of the downstream kinases. The central region of PEBPs (containing residues 60–126) might be responsible for binding to G proteins. Also, the region within hPEBP (Pro-112 to Tyr-125) is the putative

nucleotide-binding domain that overlaps with amino acid regions (Val-107 to Leu-123), sharing high homology (94%) with the sequence pattern of the G protein-coupled receptor (GPCR) signature (Kroslak et al., 2001). The 17-amino acid sequence is found in the second intracellular loop of GPCRs with a conserved acidic-arginine-aromatic triplet believed to be essential for interaction and coupling of GPCRs to heterotrimeric G proteins. This region, and the arginine, are highly conserved within specific PEBPs (Kroslak et al., 2001).

Investigators examined the previously-described hypothesis and evaluated the possible involvement of human phosphatidylethanolamine-binding protein (hPEBP) in GTP binding of G proteins and found a stimulatory effect of hPEBP on GTP binding to the cellular membranes. hPEBP might be facilitating the heterotrimeric GPCR-mediated signaling and was found to cause an approximate 38% reduction of the intracellular cAMP level, suggesting a possible role in influencing adenylate cyclase activity (Kroslak et al., 2001).

II. RAF-1 KINASE INHIBITOR PROTEIN

A novel protein, Raf-1 kinase inhibitor protein (RKIP), has been discovered that belongs to the family of PEBPs. Yeung et al. (1999) have hypothesized that the complexity of the regulation of the Ras–Raf-1–MEK [mitogen-activated protein kinase–ERK (extracellular signal-regulated kinase) kinase]–ERK module may include associations with scaffolding and regulatory proteins (Moodie et al., 1993). To isolate such proteins, they used the Raf-1 kinase domain, BXB (Bruder et al., 1992), as bait in a yeast two-hybrid screen. One clone, RKIP, bound to both kinase-active and kinase-negative BXB. At present, there are 13 identified PEBP sequences and alignment of these sequences suggesting that these proteins can be grouped into four subfamilies (PEBP-1, PEBP-2, PEBP-3, and PEBP-4). RKIP has homology to rat PEBP-3 (Simister et al., 2002), with a molecular mass of 23 kDa.

A. Properties

On the basis of the finding that RKIP is a PEBP, herein we characterize RKIP as sharing some properties with PEBP. Analysis of the crystal structure of human RKIP has suggested a possible position for this protein in the inner leaflet of the phospholipid bilayer, where it would transfer the extracellular signals to the cytoplasm (Banfield et al., 1998; Vallee et al., 2001) (Fig. 2).
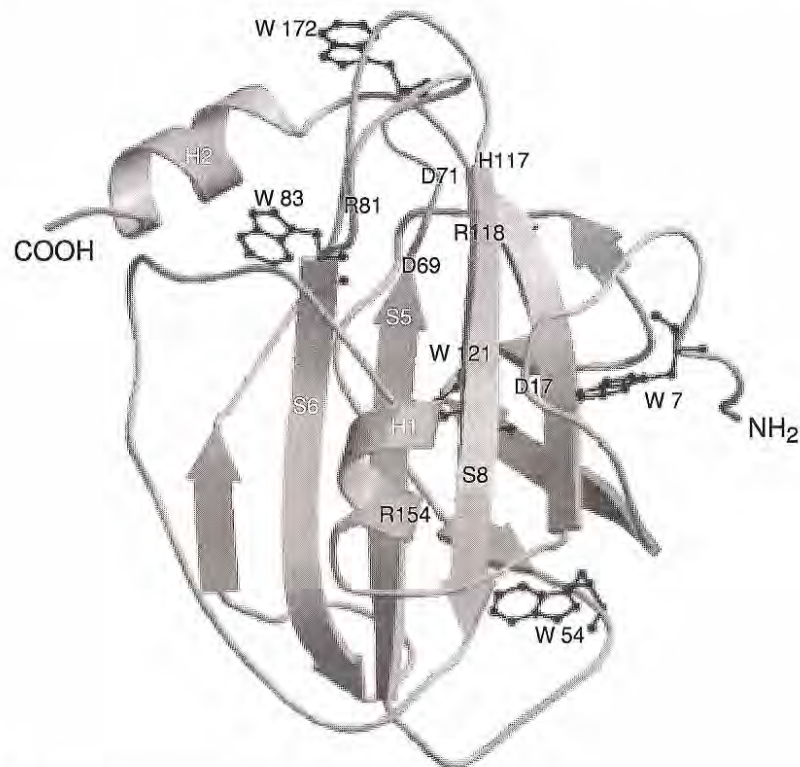
Fig. 2 Crystal structure of RKIP (Vallee *et al.*, 2001).

The RKIP crystal structure contains two virtually identical molecules (chain A with 180 residues and chain B with 185 residues in an asymmetrical unit) (Banfield *et al.*, 1998). RKIP contains four α helices and nine β strands, with unique folding different from that of any known protein (Schoentgen *et al.*, 1987). Studies based on Fourier transform (FT) infrared analysis suggest that the protein is composed mainly of antiparallel β sheets, with a tertiary structure in which most of the tryptophan residues are arranged within a hydrophobic environment (Vallee *et al.*, 2001). RKIP is located toward the negatively charged inner leaf of the plasma membrane because of its positive charge distribution (Banfield *et al.* 1998). Also, immunohistochemical studies reveal a cytoplasmic localization of RKIP; however, under different tissue culture conditions the localization is not always restricted to the cytoplasm or the inner leaflet of the plasma membrane. Further, RKIP is found to be a hydrophilic protein and with time unfolds at the air–water interface (Vallee *et al.*, 2001).

B. Functions

RKIP has been shown to be an inhibitor of both the Raf-1 and nuclear factor κB (NF-κB) signaling pathways (Yeung *et al.*, 1999, 2000, 2001) and an inhibitor of G protein-coupled receptor kinase 2 (GRK-2) (Lorenz *et al.*, 2003).

1. INHIBITION OF THE RAS–RAF-1–MEK1/2–ERK1/2 PATHWAY

Extracellular signals initiated by growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin, growth hormones, and phorbol esters bind to the transmembrane, mainly receptor tyrosine kinases (RTKs), and activate a cascade of events with resultant activation of proteins involved in cell survival (Ahn, 1993; Dong *et al.*, 2002). On binding of the ligand, RTKs dimerize with the subsequent phosphorylation of the C-terminal tyrosine residue. This provides a docking site on the receptor to which the adaptor protein, the growth factor receptor-bound protein 2 (Grb2), binds via its SH2 (Src homology 2) domain ($\sim$100 amino acids). This association is accomplished via direct binding of the SH2 domain of Grb2 to phosphotyrosine. Also, Grb2 has two SH3 domains ($\sim$60 amino acids) that associate with the proline-rich domain of the GTP exchange factor (GEF), Son of sevenless (Sos). Sos is translocated to the plasma membrane, where it interacts with and activates the G protein Ras (Genot and Cantrell, 2000). Ras is a protooncogene and activated *ras* alleles are found in a variety of tumors (Genot and Cantrell, 2000). In metazoans, the Ras–Raf-1–MEK1/2–ERK1/2 module is a signaling pathway transferring mitogenic and differentiation signals from the cell membrane to the nucleus. Ras is a guanine nucleotide-binding protein with GTPase activity and is activated by many growth factor receptors such as receptor tyrosine kinases (RTKs), GPCRs, and voltage-dependent Ca^{2+} channels. Inactive Ras binds to GDP and on phosphorylation by GEF and conversion of the GDP to GTP, Ras becomes active (Genot and Cantrell, 2000). On activation, Ras binds the serine/threonine kinase Raf-1 kinase with high affinity. Subsequently, Raf-1 is translocated from the cytosol to the cell membrane and it is activated by mechanisms that are not completely known (Morrison and Cutler, 1997). Activated Raf-1 then phosphorylates and activates MEK, a kinase that in turn phosphorylates and activates ERK, the prototypic mitogen-activated protein kinase (MAPK) (Marais and Marshall, 1996) (Fig. 3). Simultaneously, the c-Jun N-terminal kinase (JNK) pathway is activated and JNKs phosphorylate Ser-63 and Thr-73 residues of the Jun proteins, leading to their transcription (Ham *et al.*, 2000). The JNK pathway is activated by a variety of cytokines and is an active pathway in innate immune responses (Dong *et al.*, 2002). On association of Fos and Jun members, the transcription factor activation
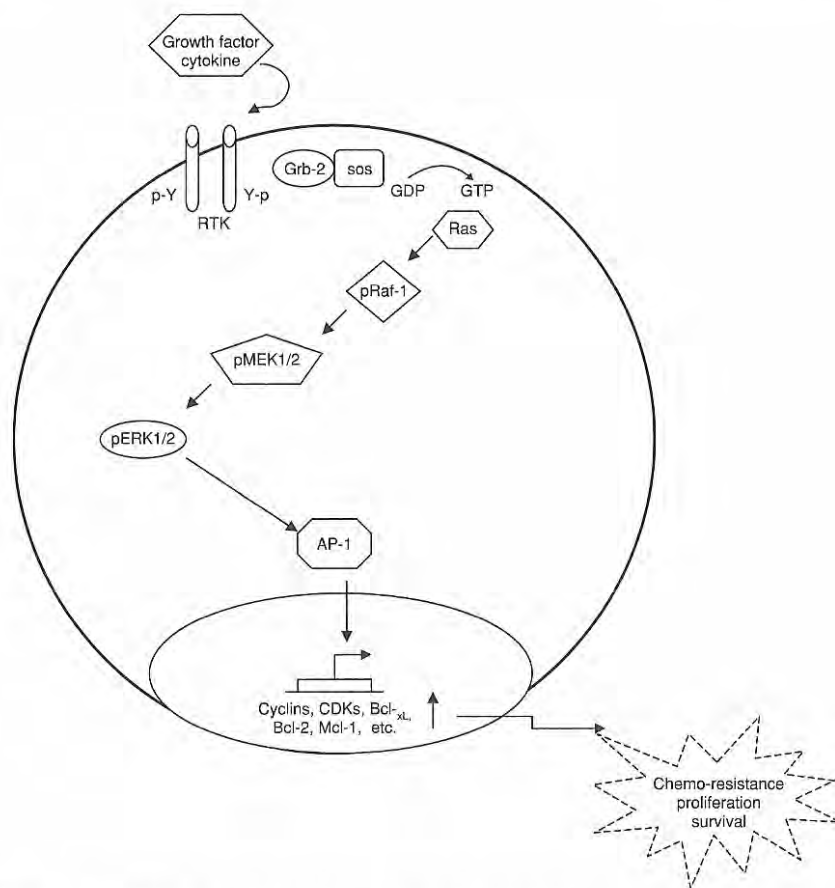
Fig. 3 The ERK1/2 signaling pathway. On binding of the ligand, receptor tyrosine kinases dimerize tyrosine residues, providing a docking site on the receptor to which the adaptor protein, the growth factor receptor-bound protein 2 (Grb2), binds via its SH2 (Src homology 2) domain. Also, Grb2 has two SH3 domains, which associate with the proline-rich domain of the GTP exchange factor (GEF) Sos (son of sevenless). Sos is translocated to the plasma membrane, where it interacts and activates the small G protein Ras. Activated Ras then activates Raf-1, which is translocated from the cytosol to the cell membrane and signals to ERK1/2 via its substrate, MEK1/2, leading to the activation of transcriptions factors (e.g., AP-1) and thus gene expression.

protein-1 complex (AP-1) is formed and becomes activated. A variety of stimuli such as cytokines and growth factors lead to the activation of AP-1, which then binds to the 12-O-tetradecanoyl phorbol-13-acetate (TPA) response element (TRE), leading to the transcription of a variety of regulatory genes responsible for cell survival and death such as *Bcl-3* (Shaulian and Karin, 2002; Whitmarsh and Davis, 1996).

Yeung *et al.* (1999) have identified RKIP as binding to Raf-1, MEK, and ERK in a two-hybrid system. RKIP interfered with the activation of the Raf-1–MEK–ERK signaling pathway both *in vitro* and *in vivo*. RKIP overexpression suppressed the ERK pathway and downregulation of RKIP had the opposite effect. A number of mechanisms have been proposed by which RKIP interferes with the Ras–Raf-1 pathway. One mechanism suggests that inhibition is a result of a conformational change in Raf-1 associated with binding to RKIP or mediated through direct steric hindrance of the Raf-1–MEK1/2 interaction (Yeung *et al.*, 2000). RKIP inhibits the phosphorylation of mitogen-activated protein kinase–ERK (extracellular signal-regulated kinase) kinase-1 (MEK-1) by Raf-1 by disrupting the interaction between the two kinases. Also, RKIP seems to specifically bind to MEK. Interaction of RKIP with MEK blocks its activation by Raf-1, leading to the suppression of both Raf-1-induced transformation and AP-1-dependent transcription. This mechanism may be due to the ability of RKIP to form ternary complexes with Raf-1, MEK, and ERK. MEK and ERK simultaneously associate with RKIP; however, Raf-1 bindings to RKIP as well as MEK are not concomitant and mutually exclusive. RKIP is capable of dissociating Raf-1–MEK complexes and acts as a competitive inhibitor of MEK phosphorylation. MEK and Raf-1 have been shown to bind to overlapping sites in RKIP. MEK and RKIP associate with different domains in Raf-1, while Raf-1 and RKIP bind to different sites in MEK. Disruption of both the Raf-1- and MEK-binding sites in RKIP is necessary to relieve RKIP-mediated suppression of the Raf-1–MEK1/2–ERK1/2 pathway. Binding of either Raf-1 or MEK is sufficient for inhibition (Yeung *et al.*, 2000). Analysis of the kinetics of MEK phosphorylation by Raf-1 showed that RKIP diminished the K_m but not the V_{max} of the reaction, which results in a competitive enzymatic inhibition (Yeung *et al.*, 2000).

2. INHIBITION OF THE NF-κB SIGNALING PATHWAY

RKIP has been shown to inhibit the NF-κB pathway (Yeung *et al.*, 2001). The transcription factor NF-κB is activated in response to a variety of stimuli such as cytokines and inflammatory responses and controls the activation of immune receptors and cytokines (Li and Stark, 2002). NF-κB is composed of a variety of protein dimers belonging to the family of proteins referred to as Rel. Members of the Rel family of proteins contain a conserved 300-amino acid N-terminal portion that is referred to as the Rel homology domain (RHD). The RHD serves as the DNA-binding site and as the dimerization region, and is required for association with the inhibitory proteins (IκB) members (Ghosh *et al.*, 1998; Karin *et al.*, 2002). The dimers are composed of Rel-A, c-Rel, and p50 and compose the cytosolic NF-κB, which is bound to IκB and remains inactive (Karin *et al.*, 2002). IκB is

composed of a series of ankyrin repeats following the N-terminal regulatory domain. Upstream, IκB kinase (IKK) is responsible for phosphorylating the IκB, which is then degraded via the proteasome system. Further, transforming growth factor β-activated kinase-1 (TAK1) and NF-κB-inducing kinase (NIK), which belong to the MAPKKK family, phosphorylate IKK (Yeung *et al.*, 2001). This leads to the release of NF-κB and its translocation to the nucleus and binding to the κB site, which promotes transcription of various genes such as those encoding cytokines, adhesion molecules, and antiapoptotic genes (Ghosh *et al.*, 1998; Karin *et al.*, 2002) (Fig. 4).
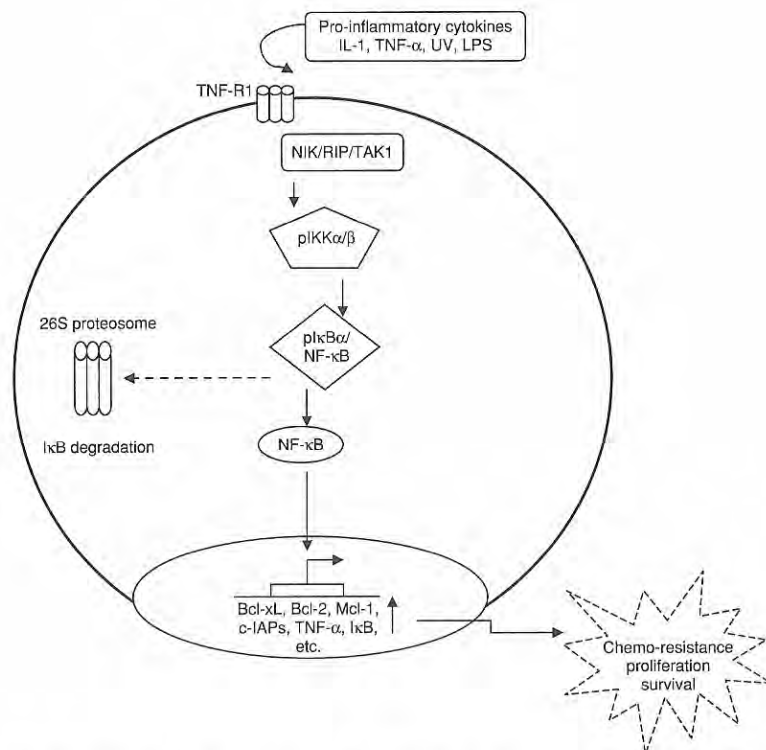
Fig. 4 The NF-κB signaling pathway. The NF-κB signaling pathway can be triggered by a plethora of stimuli such as inflammatory cytokines (IL-1), UV irradiation, and lipopolysaccharide (LPS). TNF-α is a potent activator of this pathway. The binding of TNF-α to TNF-R1 promotes rapid formation of a receptor-proximal signaling complex containing adaptor molecule TRADD, which recruits additional signaling components including RIP and NIK, which will in turn activate the IKK complex. IKK is responsible for phosphorylating IκB, which is then degraded via the proteasome system. This leads to the release of NF-κB and its translocation to the nucleus, where it leads to the expression of various genes implicated in cellular proliferation and apoptosis.

Considerable progress has been achieved in the identification of kinases that activate the IKK complexes; however, little is known about negative regulation that may interfere with these pathways (Rothwarf and Karin, 1999). Yeung *et al.* (2001) have shown that RKIP inhibits the NF-κB pathway through its interaction with upstream kinases TAK1, NIK, and IKK, both *in vitro* and *in vivo*. It was found that RKIP physically interacts with and blocks TAK1 and NIK, but not MEKK1 or NAK1, and thus inhibits the activation of these kinases, leading to inactivation of NF-κB. Further, they showed that RKIP reduces the tumor necrosis factor-α (TNF-α)-mediated activation of NF-κB. TAK1 is implicated in interleukin (IL)-1β signaling immediately upstream of NIK. RKIP has also been found to physically interact with the α and β subunits of IKK, inhibiting the phosphorylation of IκB and thus inhibiting the NF-κB signaling pathway (Yeung *et al.*, 2001). These studies suggested that RKIP acts as a brake on TNF-α and IL-1β signaling by antagonizing the activation of IKKs by NIK and TAK1 as well as by directly downmodulating the activity of the IKK complexes.

3. INHIBITION OF GRK-2

Another function has been reported for RKIP (Lorenz *et al.*, 2003). Previous studies suggested cross-talk between Raf-1 and G protein-coupled receptor kinase-2 (GRK-2) (Slupsky *et al.*, 1999). Because RKIP dissociates from Raf-1 after activation (Corbit *et al.*, 2003; Lorenz *et al.*, 2003; Yeung *et al.*, 1999, 2000), it is postulated that RKIP may be responsible for the cross-talk between Raf-1 and GRK-2. GRK-2 is the major feedback inhibitor for G protein-coupled receptors (GPCRs); it phosphorylates activated receptors, uncouples them from G proteins, and initiates their internalization (Krupuilk and Benovic, 1998; Lefkowitz *et al.*, 1998). Thus, the control of GRK-2 is vital for this kinase. These authors revealed that after stimulation of the GPCR, RKIP dissociates from Raf-1 to associate with GRK-2 and blocks its activity. This switch is triggered by protein kinase C (PKC-dependent phosphorylation of RKIP on Ser-153). By switching from Raf-1 to GRK-2, RKIP acts as a signal modifier that enhances receptor signaling by inhibiting GRK-2 and increases the signal-to-noise ratio of Raf-1-dependent signaling by suppressing basal Raf-1 activity. This helps maintain a balance between the growth-promoting pathway of Raf-1 and the inhibitory pathway of GRK-2.

C. Role of Kinases in RKIP Regulation

RKIP has been reported to be a substrate for PKCα, -βI, -βII, -γ, and -ξ (Corbit *et al.*, 2003). PKC is an enzyme activated by G protein-coupled receptors (GPCRs) and phosphorylates specific serine and threonine residues

on target proteins. PKC achieves its functions through two pathways, first by activation of the MAPK pathway and second by phosphorylation of IκB, resulting in the release of NF-κB. RKIP is phosphorylated at Ser-153 (S153) and substitution of S153 prevents most of this phosphorylation by PKC. Phosphorylation of RKIP by PKC causes release of RKIP from Raf-1 and increases ERK1/2 activation (Corbit *et al.*, 2003). Further, it was shown that phosphorylation of S153 on RKIP leads to its extension into the potential Raf-1-binding pocket, which suggests that phosphorylation at this site leads to the release of Raf-1 from RKIP and thus activation of the MAPK pathway (Corbit *et al.*, 2003). The released phospho-RKIP associates with GRK-2 and blocks its activity as described in the preceding section (Lorenz *et al.*, 2003).

D. Role of RKIP in Cell Survival

Although the molecular pathways have been partially delineated, little is known about the biological relevance of the inhibition of both the Raf-1–MEK1/2–ERK1/2 and the NF-κB pathways by RKIP. The inhibitory role of RKIP in both the NF-κB and ERK1/2 signaling pathways suggested that RKIP may regulate cell proliferation and survival. Thus, one may hypothesize that stressing cells, or treating cells with genotoxic drugs, may result in an enhanced interaction between RKIP with components of these pathways and may shift the balance from proliferation and cell survival toward inhibition of proliferation and signaling for apoptosis induction. Further, the induction of RKIP expression by these stimuli may enhance the antiproliferative and apoptotic effects. In the following sections, we summarize experiments that explored this hypothesis, utilizing malignant cell lines treated with chemotherapeutic drugs used in the treatment of such malignancies and a non-Hodgkin's lymphoma (NHL) tumor model following treatment with rituximab (anti-CD20). In both cases, upregulation of RKIP expression was noted.

1. ROLE OF RKIP IN CYTOTOXICITY BY CHEMOTHERAPEUTIC DRUGS IN TUMOR CELL LINES

On the basis of the findings that RKIP expression interferes with the survival pathways and the fact that most drugs interfere with the same survival pathways, it is possible that RKIP regulates apoptosis in response to chemotherapeutic drugs. Studies of various cancer cell lines have shown an upregulation of RKIP on treatment with an array of drugs. This suggested that one potential mechanism by which various drugs lead to apoptosis might be through the induction of RKIP. Experiments with the human

prostate carcinoma cell line DU145 have shown that these cells undergo extensive apoptosis after treatment with the topoisomerase I inhibitor 9-nitrocamptothecin (9NC) (Chatterjee *et al.*, 2004) (Fig. 5A). However, treatment of the DU145-resistant cell line RC1 with 9NC does not result in apoptosis (Fig. 5B). Given the association between RKIP and NF-κB activity (Fig. 5C, D), studies were designed to investigate whether there was a correlation between the levels of RKIP in 9NC-sensitive and 9NC-resistant cell lines. Indeed, in parental DU145 cells, 9NC triggered a significant induction of RKIP, an effect that was not observed in RC1 cells (Fig. 5C), implying that induction of RKIP may be necessary for these cells to undergo 9NC-triggered apoptosis. This hypothesis was confirmed when it was demonstrated that blocking RKIP [by antisense small interfering RNA (siRNA) approaches] abrogated apoptosis in DU145 cells in response to 9NC. Moreover, ectopic expression of RKIP in RC1 cells sensitized these cells to 9NC-triggered apoptosis (Fig. 5E). Studies were also performed to evaluate whether 9NC can induce expression of RKIP in androgen-independent PC3 cells. A significant increase in RKIP expression was observed in these cells after treatment with 9NC (data not shown). In addition, exposure to other genotoxic stimuli such as etoposide or cisplatin resulted in the induction of RKIP expression in the DU145 model (Fig. 5E). Similar results were also found in drug-sensitive and drug-resistant breast carcinoma cell lines (data not shown). The molecular mechanism(s) responsible for the induction of RKIP protein by the various cytotoxic agents is not yet known. Nonetheless, the direct correlation of RKIP induction and sensitivity to drug-induced apoptosis suggests that RKIP overexpression or induction may be necessary for apoptosis in tumorigenic prostate cancer cell lines after exposure to clinically relevant chemotherapeutic compounds (Chatterjee *et al.*, 2004).

2. RKIP INDUCTION BY RITUXIMAB AND SENSITIZATION OF NHL TO CHEMOTHERAPEUTIC DRUGS

Rituximab (Rituxan, IDEC-C2B8) is the first Food and Drug Administration-approved chimeric monoclonal antibody (mAb) for the treatment of various types of lymphomas that is specifically directed against the B cell-restricted marker CD20 (Reff *et al.*, 1994). Its primary use has been for the treatment of follicular and low-grade NHL, but it is now in wide use for an assortment of B cell cancers and proliferative disorders (Coiffier, 2003; Huhn *et al.*, 2001). Previous findings demonstrated that rituximab sensitizes drug-resistant NHL cells to drug-induced apoptosis (Alas and Bonavida, 2001; Demidem *et al.*, 1997). In addition, rituximab also inhibits NF-κB and AP-1 DNA-binding activities in NHL cells resulting in the downregulation of Bcl-x$_L$ expression and sensitization to drugs [e.g., paclitaxel, doxorubicin,
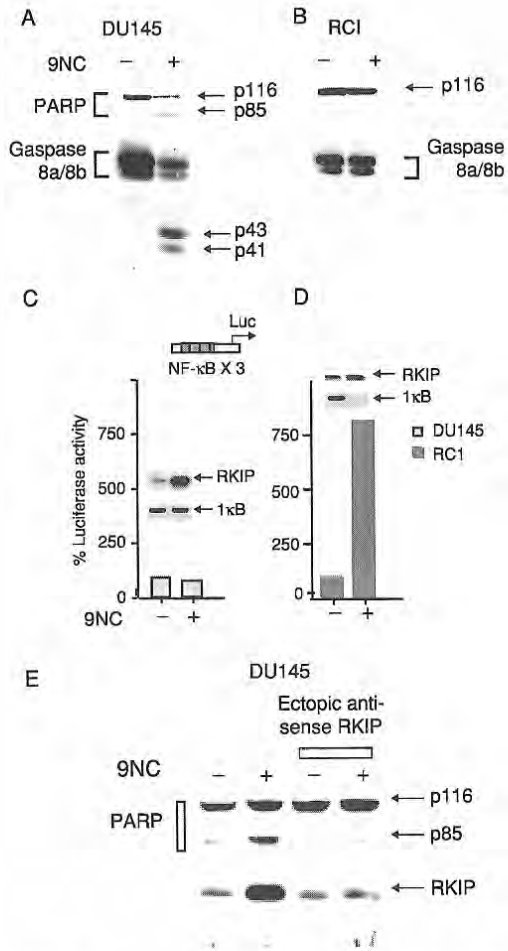
Fig. 5 Regulation of RKIP expression by 9NC in prostate cell lines. Extracts were prepared from DU145 cells and RC1 cells untreated (–) or treated with 9NC for 24 h (+). Extracts were prepared for Western blot analysis to examine (A) poly(ADP-ribose) polymerase (PARP) and procaspase-8 and (B) the expression of RKIP, IκB, and NF-κB activity from cells transfected with an expression vector for RKIP (CMV-HA-RKIP) and an NF-κB reporter (NF-κB×3Luc). Twenty-four hours after transfection, cells were treated with 9NC for an additional 24 h. Extracts were prepared from DU145 cells transfected with (C) antisense-HA-RKIP and examined for PARP and RKIP, and from DU145 cells transfected with (D) CMV-HA-RKIP and IκB-SR (IκB super repressor) and examined for RKIP. DU145 cells treated with cisplatin and etoposide and examined for PARP cleavage and RKIP expression (E).

and cisplatin (CDDP)] (Jazirehi *et al.*, 2000, 2003a,b). Our laboratory has been engaged in delineating the intracellular signal transduction pathways initiated on treatment of NHL B cell lines with rituximab (Alas and Bonavida, 2001; Demidem *et al.*, 1997). Rituximab exerts a cytostatic effect on NHL cell lines (Table II) and selectively downregulates the expression of antiapoptotic gene products Bcl-2 and Bcl-x$_L$, thus sensitizing the drug-refractory tumor cells to the apoptotic effects of an array of chemotherapeutic agents (Alas and Bonavida, 2001; Jazirehi *et al.*, 2003a,b). Because Bcl-2 and Bcl-x$_L$ are regulated by NF-κB and AP-1 (downstream of the Raf-1–MEK–ERK pathway) (Jazirehi *et al.*, 2003a, 2004a), and because RKIP has been shown to regulate AP-1 (Yeung *et al.*, 1999), we examined whether rituximab-mediated inhibition of NF-κB and AP-1 may be due, in part, to RKIP interference. Our hypothesis is that rituximab, via direct or indirect interaction, interferes with major signaling pathways implicated in cellular proliferation such as the NF-κB and the ERK1/2 pathways. Because RKIP is at the core of this network, acting as a bridging molecule between these signaling modules, we investigated whether it was involved in the signaling by rituximab. Rituximab treatment significantly reduces the proliferation rate of NHL cell lines (Emmanouilides *et al.*, 2002). Moreover, time kinetics studies revealed that rituximab downregulates the phosphorylation-dependent state of IκBα and ERK1/2 and upregulates the expression levels of RKIP with similar kinetics (Jazirehi *et al.*, 2004a). These findings corroborate our hypothesis and suggest that rituximab-mediated upregulation of RKIP inhibits the ERK1/2 and NF-κB signaling pathways, which

Table II Inhibition of Proliferation of Non-Hodgkin's Lymphoma B Cells by Rituximab[a]

	Total viable cell no. (×10⁴)	Percent inhibition of cell proliferation
Ramos	254 ± 3.5	
Ramos + rituximab	149 ± 2.8	41.34
Raji	146 ± 4.2	
Raji + rituximab	111 ± 7.1	23.98
Daudi	129 ± 4.9	
Daudi + rituximab	73 ± 4.9	43.42
2F7	150 ± 2.9	
2F7 + rituximab	116 ± 2.1	22.67

[a]Tumor cell lines (1 × 10⁶ cell/ml) were either left untreated or pretreated with rituximab (20 μg/ml for 24 h). At the end of the incubation period an aliquot of the cell suspension was mixed with an equal volume of 4% trypan blue dye solution and total viable cell number and percent inhibition of proliferation were measured by light microscopy. The results are presented as means ± SD of two independent experiments (Emmanouilides *et al.*, 2002).

results in inhibition of Bcl-2 and Bcl-x$_L$ expression and reduction in the proliferation rate of NHL cells (see diagram in Fig. 6). These results establish a correlation between RKIP expression and the enhanced sensitivity of tumor cells to apoptosis-inducing stimuli. The findings with rituximab in
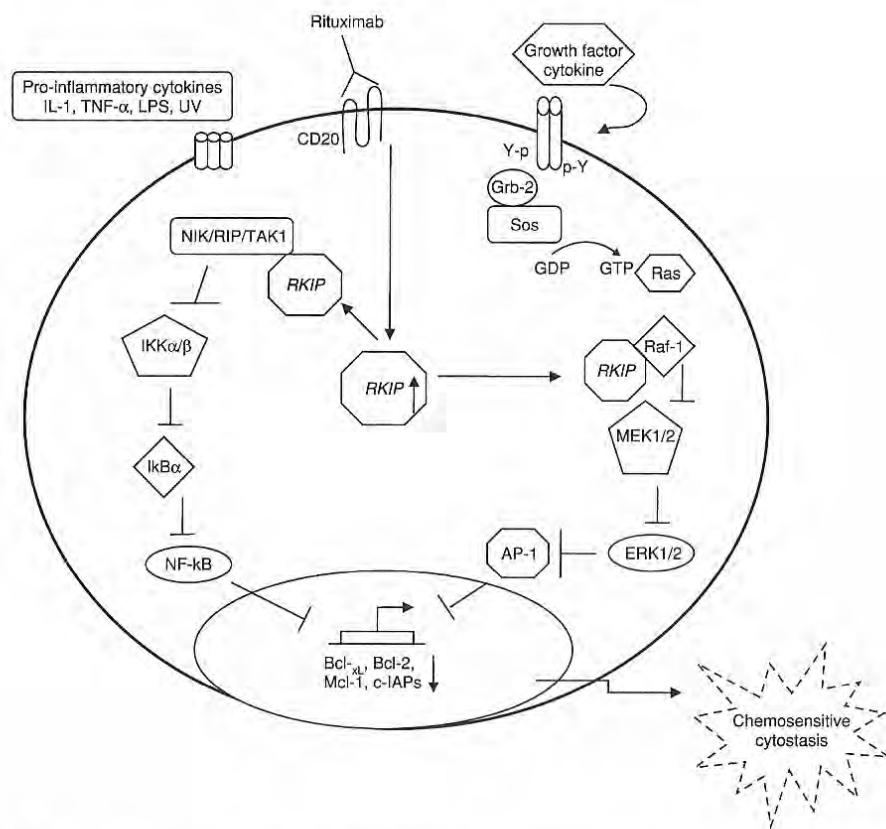
Fig. 6 Proposed model of the interaction of RKIP with the NF-κB and ERK1/2 signaling pathways in B cell lymphoma treated with rituximab. The activation of NF-κB and Ras pathways contributes to proliferation, growth, and resistance to apoptosis of B cells. These events are mediated via the transcriptional regulation of modulators of apoptosis such as the Bcl-2 (Bcl-2, Bcl-x$_L$) and IAP (c-IAP-1, c-IAP-2) family members. Previous work in our laboratory has demonstrated that the anti-CD20 mAb rituximab interferes with these signaling pathways by decreasing the phosphorylation-dependent state, thereby deactivating IκBα and ERK1/2, major components of the NF-κB and MAPK pathways, respectively. This in turn results in selective downregulation of the antiapoptotic gene products Bcl-x$_L$ in Bcl-2-deficient Ramos B cells and subsequent sensitization of the cells to the apoptosis induced by a wide array of chemotherapeutic drugs. Our findings indicate that rituximab upregulates the expression of RKIP at the protein level with the same time kinetics as deactivation of IκBα and ERK1/2. Therefore, induction of RKIP by rituximab might interfere with major antiapoptotic signaling pathways.

triggering RKIP expression and chemosensitization in NHL are consistent with the findings of Chatterjee *et al.* (2004) concerning the role of RKIP in drug-induced apoptosis (Jazirehi *et al.*, 2003a, 2004a).

E. Role of RKIP in the Regulation of Cell Signaling for Apoptosis

1. APOPTOSIS

Apoptosis or programmed cell death (PCD) is inherently present in most cells, including tumor cells, and is activated by the appropriate stimulus. Uncontrolled activation of apoptosis may lead to a variety of diseases such as cancer and autoimmune diseases (Thompson, 1995). Phenotypically, apoptosis is associated with cytoskeletal disruption, cellular shrinkage, nuclear and chromosomal DNA condensation and fragmentation, membrane blebbing, activation of endonucleases, as well as the formation of membrane-bound apoptotic bodies (Klaus *et al.*, 1996; Sakahira *et al.*, 1999). The two major apoptotic pathways initiated by activation of T lymphocytes are the granule exocytosis pathway, by activation of perforin and granzymes, and the death receptor signaling pathways, involving death receptor ligands such as tumor necrosis factor-α (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) (Shresta *et al.*, 1998) (Fig. 7). The granule exocytosis pathway is initiated by direct interaction between lymphocytes and tumor cells and involves the recognition of T cell receptor (TCR)–major histocompatibility complex (MHC), leading to the release of cytotoxic granules such as perforin. On perforin polymerization, pores are formed on the target cell membrane, which will allow the passage of granzymes from lymphocyte to the target cell, leading to apoptosis. The death receptor signaling pathway is triggered by binding of the death-inducing ligands to their cognate receptors and subsequent induction of apoptosis (Ashkenazi and Dixit, 1998).

The regulation and execution of apoptotic cell death are carried out by a family of cysteine proteases with aspartic acid specificity known as caspases. Caspases are present in living cells as inactive zymogens, and their activation occurs through autocatalytic processing by caspase cascades. Caspases are divided into initiators (e.g., caspase-8, -9, and -10) and effectors/executioners (e.g., caspase-3, -6, and -7). On the basis of the pattern of caspase cascade activation, two types of cells have been characterized so far (Scaffidi *et al.*, 1998; Thornberry and Lazebnik, 1998): in type I cells, the caspase cascade is triggered on the oligomerization of cell surface death receptors and undergoes a sequential activation of the initiator caspase, caspase-8, to the principal mediator of apoptosis, caspase-3. An alternative
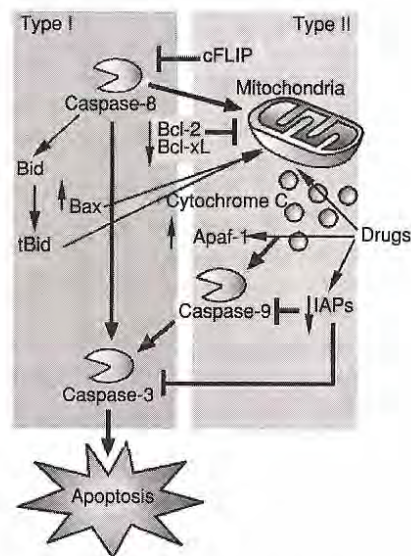
Fig. 7 Type I and II apoptotic signaling pathways. On binding to their cognate receptors, the death-inducing ligands (TRAIL, TNF-α, Fas ligand) induce the formation of the receptor-proximal death-inducing signaling complex (DISC) composed of the adapter molecule FADD (Fas-associated death domain protein) and caspase-8, which will be recruited to the cytoplasmic domain of the receptors [~80-amino acid conserved sequence called the death domain (DD)]. Depending on the amount of caspase-8, the apoptotic stimuli either directly induce caspase-8 autocleavage/processing (by yet unknown mechanisms), which leads to caspase-3 activation and cleavage of death substrates [e.g., poly(ADP) ribose polymerase; PARP] with subsequent induction of apoptosis or, alternatively, in the event that cells lack sufficient amounts of caspase-8, the apoptotic stimuli (e.g., most chemotherapeutic drugs) utilize the type II apoptotic signaling pathway involving mitochondria and the Bcl-2 family members. In the cytosol, caspase-8 will cleave the proapoptotic Bid. The caspase-cleaved fragment of Bid (truncated Bid; tBid) will then migrate to and reside in the mitochondrial outer membrane, where, in association with other proapoptotic molecules (e.g., Bax, Bad, Bcl-x$_S$), it will induce the formation of mitochondrial permeability transition pore (PTP), which will lead to mitochondrial collapse and a decrease in mitochondrial transmembrane potential ($\downarrow\Delta\psi_m$). The integrity of mitochondria is preserved by the protective effects of antiapoptotic Bcl-2 family members (e.g., Bcl-2, Bcl-x$_L$, Mcl-1). Mitochondrial destabilization will facilitate the unidirectional release of the apoptogenic molecules (cytochrome c and Smac/DIABLO) into the cytosol. Cytochrome c, in the presence of the adaptor molecule apoptosis protease-activating factor-1 (Apaf-1), dATP/ATP, and caspase-9, will participate in the formation of the apoptosome complex. The formation of the apoptosome complex expedites caspase-9 activation. Simultaneously, Smac/DIABLO will physically associate with the cellular inhibitors of apoptosis (c-IAP) family members (c-IAP-1, c-IAP-2, XIAP, and survivin), thereby removing the inhibitors of caspase activation. Through autocatalytic processing, caspase-9 becomes activated and in the absence of c-IAPs utilizes caspase-3, -6, and -7 as substrates and apoptosis ensues. This is the converging point of the type I and II pathways.

apoptotic pathway is seen in type II cells and involves mitochondrial damage and caspase-9 activation. On apoptotic stimuli, cytochrome c is released from the mitochondrial inner membrane and binds to the adaptor molecule Apaf-1, which recruits procaspase-9 and forms the apoptosome complex (cytochrome c–Apaf-1–caspase-9) that results in the activation of caspase-9. Active caspase-9 then causes the activation of caspase-3 and -6. The activation of effector caspase, caspase-3, is the merging point of the two caspase cascade pathways. Activated (processed) caspase-3 cleaves death substrates and leads to the apoptotic cell death (Bossy-Wetzel *et al.*, 1999; Ng and Bonavida, 2002; Scaffidi *et al.*, 1998; Thornberry and Lazebnik, 1998).

Although the previously-described findings demonstrate that RKIP negatively regulates the ERK1/2 and NF-κB signaling pathways and that these in turn regulate the apoptotic signaling pathways, it is not clear at present how RKIP may regulate apoptosis-inhibiting stimuli. On the basis of the inhibitory effect of RKIP on the ERK1/2 and NF-κB pathways, it is likely that inhibition of these pathways by RKIP will result in modifications in the expression profile of gene products, which primarily regulate cell survival and protect the cell from apoptosis. These two pathways and their corresponding transcription factors (NF-κB and AP-1) have been shown to regulate several apoptotic regulatory proteins such as Bcl-2, Bcl-x$_L$ (Jazirehi *et al.*, 2003a), Mcl-1, cellular inhibitors of apoptosis (c-IAP), TNF-α, and IκBα. Thus, it seems logical to speculate that the linkage between increased RKIP expression and enhanced sensitivity to apoptosis is a direct result of alterations in the expression pattern of apoptosis-related gene products.

2. ROLE OF RKIP INHIBITION OF NF-κB AND THE REGULATION OF APOPTOSIS

NF-κB, a member of the Rel transcription factor family, participates in the mediation of many biological activities such as inflammation, immune response, cell proliferation, and programmed cell death. NF-κB normally resides in the cytoplasm in association with its inhibitor IκB. Through noncovalent association, IκB masks the nuclear localization signal of NF-κB, thereby preventing NF-κB nuclear translocation. IκB is phosphorylated by IKK complex, ubiquitinated, and degraded in the proteasome; thereafter NF-κB is translocated to the nucleus, where it participates in transcriptional regulation of a wide array of genes with diverse biological activities (Karin *et al.*, 2002). The genes regulated by NF-κB include inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α), antiapoptotic c-IAPs (c-IAP-1 and -2), and Bcl-2 (Bcl-2, Bcl-x$_L$, and Mcl-1) family members and those that negatively regulate NF-κB itself (e.g., IκBα; Karin *et al.*,
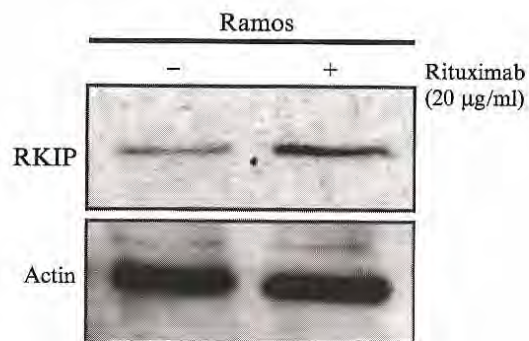
Ramos

Fig. 8 RKIP induction in Ramos cells by rituximab. Ramos cells were grown either in complete medium or in complete medium supplemented with rituximab (20 μg/ml) for 24 h. At the end of the incubation period, the cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and total cell lysates (40 μg) were subjected to immunoblot analysis using anti-RKIP polyclonal antibody. Levels of β-actin were determined to confirm equal loading of samples.

2002). Thus, NF-κB can alter the expression of proapoptotic and antiapoptotic genes, leading to various biological outcomes depending on the stimulus and cell type. Our findings demonstrate that upregulation of RKIP by rituximab in the NHL Ramos B cell line inhibits the NF-κB pathway, thus decreasing the expression of antiapoptotic genes regulated by NF-κB (e.g., Bcl-x$_L$ and Bcl-2) and shifting the balance from antiapoptotic to proapoptotic signaling pathways. Hence, the drug-resistant Ramos B cell line becomes sensitized to drug-induced apoptosis (Jazirehi *et al.*, 2004b) (Fig. 8).

3. ROLE OF RKIP INHIBITION OF AP-1 IN THE REGULATION OF APOPTOSIS

AP-1 transcription factors are involved in both the survival and apoptotic pathways. Some AP-1 proteins such as c-Jun are encoded by immediate-early genes activated by a variety of stress-related stimuli, and some are posttranslationally modified to increase the transcription activity (Ventura *et al.*, 2003). Studies have revealed various roles for this family of proteins such as a role for c-Jun in induction during ultraviolet (UV)-induced apoptosis (Shaulian and Karin, 2002) as well as studies showing that c-Jun protects against UV-induced cell death (Wisdom, 1992). There are two suggested models as to how AP-1 participates in and affects cell death and survival. First, AP-1 induction will lead to the transcription of various genes such as FasL and Bim, whose products will determine whether a cell will survive or undergo apoptosis (Shaulian and Karin, 2002). The second model

proposes that AP-1 functions as a homeostatic regulatory factor that maintains cells in a defined proliferative state. Environmental factors might change AP-1 activity and it is the balance between survival and apoptotic genes that determines whether cells undergo apoptosis or survive due to AP-1 activity (Shaulian and Karin, 2002). It has been reported that RKIP inhibits the Raf-1–MEK–ERK pathway, which regulates the activity of AP-1 (Yeung *et al.*, 1999). Therefore, it is expected that inhibition of AP-1 activity by RKIP will result in modification of the expression of antiapoptotic proteins, which would diminish the antiapoptotic activity of these molecules, and the cells would become more sensitive to apoptotic signaling by various stimuli. Indeed, in our studies with rituximab, we have found that treatment of Ramos NHL with rituximab resulted in inhibition of the Raf-1–MEK–ERK pathway and downstream inhibition of AP-1 DNA-binding activity (Jazirehi *et al.*, 2000, 2004a). Inhibition of AP-1 resulted in the inhibition of Bcl-x$_L$ transcription and sensitized the cells to drug-induced apoptosis. RKIP induction by rituximab was responsible in large part for inhibition of the Raf-1–MEK–ERK pathway.

Clearly, RKIP inhibits the NF-κB and ERK1/2 pathways and inhibition of either one of these pathways by itself might be sufficient to trigger execution of the apoptotic signaling. This implies that simultaneous inhibition of the ERK1/2 and NF-κB pathways on RKIP induction and crippling of the transcriptional activity of NF-κB and AP-1 might amplify apoptosis signaling. Although rituximab induced RKIP expression and ERK1/2 inhibition, we were unable to detect significant apoptosis in NHL cells by rituximab treatment alone. However, these cells exhibited enhanced sensitivity to drug-induced apoptosis (Jazirehi *et al.*, 2004a,b). Similar findings were also observed in prostate cancer cell lines in which transfection with RKIP sensitized drug-resistant tumor cells to drug-induced apoptosis (Chatterjee *et al.*, 2004). In contrast, overexpression of RKIP in drug-sensitive and drug-resistant cell lines resulted in the induction of apoptosis in the absence of drugs. Thus, it appears that the biological outcomes on RKIP induction (chemosensitization versus direct induction of apoptosis) might be cell type specific and/or depend on the differentiation stage and the activation status of the signaling molecules in the cells.

4. ROLE OF RKIP REGULATION OF GRK-2 IN THE REGULATION OF APOPTOSIS
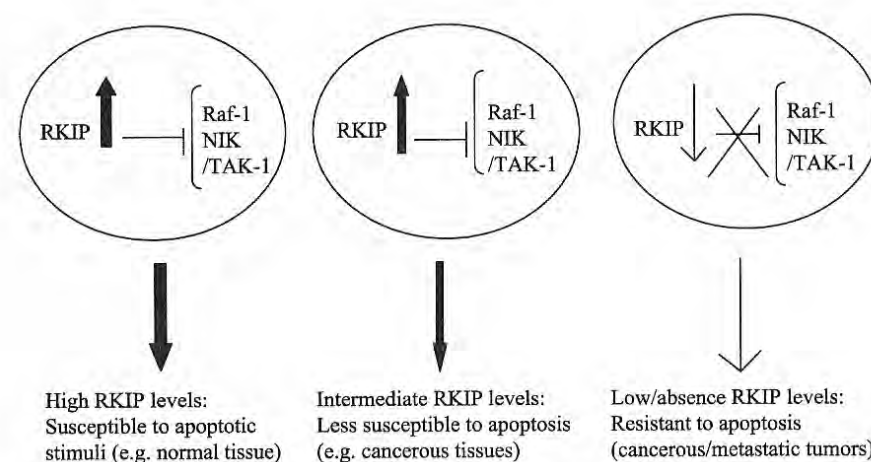
We have discussed the novel role of RKIP in the regulation of GRK-2 by inhibiting its activity. Activation of GPCRs results in the dissociation of RKIP from Raf-1, and its phosphorylation by PKC on Ser-153, and association with GRK-2 to block its activity. Thus, it may be postulated in cancer cells that in the absence of activation by G protein-coupled receptors, RKIP

may be primarily associated with Raf-1 and IKK and regulating the survival pathways, depending on the level of RKIP expression. Tumor cells will undoubtedly be selected for low expression of RKIP to maintain survival and the induction of RKIP expression will shift the balance to cell death pathways, as discussed previously, with rituximab and chemotherapeutic drugs. However, tumor cells that are constitutively activated will respond to activation of GPCRs and will utilize RKIP to maintain the stimulus and activation in the cells and increase the threshold of drug/immune-induced apoptosis.

F. Transcriptional Regulation of RKIP Expression

The previously-described findings demonstrate the important role of RKIP in the regulation of both cell survival and apoptosis. This suggests that transcription, translation, and posttranslational regulation of RKIP in various cell lines can be important determinants of cell fate in response to noxious stimuli. Therefore, the expression of RKIP must be tightly regulated at the translational and posttranslational levels. The mechanisms by which RKIP expression is regulated at different levels in different cell types and in different stages of differentiation have not been examined. We evaluated the amino acid sequence of RKIP protein and used TESS master analysis (Schug and Overton, 1997) to obtain the nucleotide sequence of the RKIP gene located on chromosome 12. After examining the promoter sequence, we found putative transcription factors that might regulate the expression of RKIP. Computer database analysis of RKIP has revealed AP-1, clusters of SP-1, and Ying and Yang-1 (YY1) consensus binding sites (Fig. 9).

It appears that the transcriptional regulation of RKIP might be influenced by the activity of transcriptional activators and/or repressors. The transcriptional activity of the activators and repressors is governed by different mechanisms as well as by the different levels of their expression. Therefore, it is possible that, for instance, overexpression of the transcription repressor YY1 may dominate that of the transcriptional activators, which will minimize the expression levels of RKIP. Removal of this inhibitory effect should theoretically enhance RKIP expression. Indeed, treatment of prostate carcinoma cell lines with inhibitors for NF-κB or the transcription repressor YY1 resulted in upregulation of RKIP expression (Huerta-Yepez *et al.*, 2004). The reverse may also be true. Distinguishing the roles of each of these transcription factors in the overall regulation of RKIP expression is a complex undertaking. However, we have begun to dissect the role of the transcription factors in the regulation of RKIP transcription in different cell lines. The presence of AP-1-binding sites on the RKIP

Fig. 9 Nucleotide sequence of the promoter region of RKIP. The underlined sequences represent putative transcritpion factors binding sites.

promoter suggests a possible regulatory role for this factor in the transcription of RKIP. Activation of the AP-1 family of transcription factors occurs through the involvement of the MAPK pathways (Ventura *et al.*, 2003). These transcription factors are involved in various cellular processes such as proliferation, survival, and apoptosis. Another possible mechanism of RKIP transcriptional regulation might also be through Specific protein-1 (Sp1) transcription factors. Sp1 belongs to the family of Sp/XKLF zinc finger proteins, which regulate a variety of genes involved in survival and apoptosis through binding to G/C-rich *cis*-regulatory sequences and interaction with the transcription machinery (Marco *et al.*, 2003). Sp1 has been shown to play a role in the regulation of tissue factor, insulin-like growth factor-binding protein-1, and plasminogen activator inhibitor-1 (Krikun and Lockwood, 2002). Sp1 and Sp3 are known as oxidative stress-induced transcription factors in cortical neurons, where they regulate the survival of the neurons (Ryu *et al.*, 2003). Also, Sp1 has been shown to regulate the activation of several genes as well as to export proteins after TGF-β treatment (Verrecchia *et al.*, 2001); thus, the presence of Sp1-binding sites on the RKIP promoter might suggest that Sp1 might regulate RKIP expression. Finally, a mechanism of RKIP regulation might be through the YY1 transcription factor. The nuclear protein YY1 (δ, NF-E1, UCRBP, CF1) is a highly conserved 68-kDa zinc finger transcription factor (located on

14q32). It acts as both a transcriptional repressor as well as an activator (Austen *et al.*, 1997). YY1 is a stable phosphoprotein expressed ubiquitously regardless of cell cycle stage or differentiation status (Austen *et al.*, 1997), which suggests that the activity of YY1 is regulated at the posttranslational level, possibly through interaction with other proteins. Further, it participates in cell survival by repressing genes such as *fas* (Garban and Bonavida, 2001). Theoretically, YY1 binding to RKIP could repress the transcription of RKIP. Thus, the inhibition of YY1 DNA-binding activity would allow RKIP to be expressed, which will interfere with the survival pathways. We show that treatment of 2F7 NHL with rituximab inhibits YY1 activity and induces RKIP expression, consistent with the role of transcription repressor YY1 in the regulation of RKIP (Vega *et al.*, 2004). The mechanism(s) by which rituximab or drugs might trigger this process are yet to be unraveled. We further suggest a possible role of cytokines in the constitutive regulation of RKIP expression.

III. SIGNIFICANCE OF RKIP IN CANCER

Regulation of cell survival and apoptotic pathways by RKIP suggests that this protein may play a pivotal role in tumor progression and may represent a novel prognostic marker of apoptosis. The lack and/or low levels of RKIP expression in tumor cells would theoretically allow progression of the disease and resistance to apoptosis-inducing stimuli. This is consistent with the role of RKIP in regulating the survival of cells. Thus, cells that overexpress RKIP will experience interference in survival pathways, rendering the cells susceptible to apoptotic stimuli. Cells that express moderate levels of RKIP (e.g., cancer cells) will be prone to cell survival and less susceptible to apoptotic stimuli. In contrast, cells that lack RKIP (advanced cancer and metastatic tumors) will be highly resistant to apoptotic stimuli (see schematic diagram in Fig. 10). This rationale is consistent with the observations of Chatterjee *et al.* (2004) and Jazirehi *et al.* (2003a) discussed in Section II.E.3. In addition, findings on RKIP expression in cancer metastasis support the previously-described rationale. Hence, Fu *et al.* (2002, 2003) used gene array analysis to identify genes whose expression changes during the transition from nonmetastatic (LNCaP) prostate carcinoma cells to metastatic prostate cancer cells derived from LNCaP (C4-2B). They found that the expression of one gene, that encoding RKIP, was lower in the metastatic cell line than in the nonmetastatic cell line. The expression of RKIP was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis, with C4-2B cells having four to five times less RKIP than the parental LNCaP cells. The investigators

High RKIP levels: Susceptible to apoptotic stimuli (e.g. normal tissue)

Intermediate RKIP levels: Less susceptible to apoptosis (e.g. cancerous tissues)

Low/absence RKIP levels: Resistant to apoptosis (cancerous/metastatic tumors)

Fig. 10 Regulation of apoptosis in cancer cells by RKIP. This scheme illustrates the possible role of RKIP expression in cancer cell and response to apoptotic stimuli. Overexpression of RKIP will interfere with the survival pathway (the Raf-1 and NF-κB pathways) and renders the cells susceptible to apoptotic stimuli. Cells that express moderate levels of RKIP (e.g., cancer) will be prone to cell survival and less susceptible to apoptotic stimuli. In contrast, cells that lack RKIP (advanced cancer and metastatic tumors) will be highly resistant to apoptotic stimuli.

suggest RKIP function as a suppressor of metastasis. RKIP is the thirteenth metastasis suppressor described in the literature (Kauffman *et al.*, 2003). Metastatic suppressors may be independent prognostic markers. There are suggestions that metastatic suppressor genes are not mutated but instead are differentially expressed (Steeg *et al.*, 2003) at the protein translational level, or are affected by mechanisms that involve gene multiplication, histone acetylation, and mRNA or protein stability (see commentary by Welsh and Hunter, 2003). Studies by Fu *et al.* (2003) have demonstrated that the low levels of RKIP mRNA and protein are correlated with the metastatic potential of human C4-2B prostate cancer cells when compared with parental nonmetastatic LNCaP cells. Moreover, overexpression of RKIP in C4-2B cells decreased cell invasion *in vitro* and progression of lung metastases *in vivo*. In addition, increased levels of RKIP were associated with decreased vascular invasion of the primary tumor, with no effect on primary tumor growth in mice (Fu *et al.*, 2003). These results suggest that RKIP does not affect the tumorigenic properties of these prostate cells but may represent a clinically significant suppressor of metastasis by decreasing vascular invasion (Fu *et al.*, 2003). It will be important to evaluate whether the expression of RKIP is associated with the progression and clinical outcome of other cancers to determine whether molecular targeted therapy for this protein is rational.

IV. CONCLUDING REMARKS

Molecular targeted therapies are becoming a prominent feature of modern oncology, the ultimate therapeutic goal of which is to trigger tumor-selective programmed cell death (apoptosis). Therefore, the molecular mechanisms responsible for such death are of obvious importance in determining the efficacy of specific treatments and chemotherapeutic compounds. In drug-curable malignancies, apoptosis is a prominent mechanism associated with the induction of tumor remission. Further, the expression of apoptosis modulators within a tumor appears to correlate with its sensitivity to traditional therapies. The direct correlation between RKIP expression and the regulation of apoptosis by drugs and immunotherapy raises the possibility that upregulation of RKIP by drugs is one of the mechanisms that either directly induces apoptosis in response to drugs or indirectly sensitizes the malignant cells to apoptosis induced by chemotherapeutic agents and immunotherapeutics. Further, the role of RKIP in regulating the various signaling pathways (Raf-1–MEK–ERK, IKK-IKB-NF-κB, GRK-2) suggests that these pathways are potential targets for intervention. Further, in future it will be important to delineate its transcriptional regulation and identify agents that selectively induce and/or inhibit RKIP in cancer cells. The demonstration that RKIP functions as a metastatic suppressor is clinically significant not only for its importance as a prognostic marker but also because it identifies new targets for therapeutic intervention in the regulation of metastasis. More comprehensive understanding of how RKIP is regulated and expressed in tumor samples and how it directly affects apoptotic and signal transduction pathways will allow its optimal utilization for therapeutic advantage in human cancers.

ACKNOWLEDGMENTS

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Prognostic Significance of Serum Osteoprotegerin Levels in Patients with Bladder Carcinoma

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BACKGROUND. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) plays an important role in the process of lymphocyte-mediated cytotoxicity against malignant cells. Osteoprotegerin (OPG) is a soluble decoy receptor for TRAIL, and circulating OPG has been implicated in the protection of cells from TRAIL-mediated apoptosis. Thus, OPG may protect tumor cells from lymphocyte-mediated cytotoxicity and, as a result, contribute to tumor progression. In the current study, the authors investigated this hypothesis in patients with bladder carcinoma.

METHODS. Serum OPG levels for 185 patients with bladder carcinoma were determined using an enzyme-linked immunosorbent assay. These levels then were assessed for potential correlations with various disease characteristics and outcome measures.

RESULTS. The mean serum OPG concentration in patients with bladder carcinoma was approximately 3 times greater than the mean concentration in healthy individuals, and among patients with bladder carcinoma, higher tumor stage and grade were found to be associated with increased serum OPG levels. Within the subpopulation of patients with superficial bladder carcinoma, after a follow-up period of 5 years, those who had low serum OPG levels tended to have a longer postoperative tumor-free interval compared with those who had high serum OPG levels. Furthermore, among patients with muscle-invasive bladder carcinoma, the 5-year disease-specific survival rate was greater for those who had low serum OPG levels than for those who had high serum OPG levels.

CONCLUSIONS. To the authors' knowledge, the current study is the first to demonstrate that serum OPG concentration is correlated with both tumor stage and tumor grade and that elevated serum OPG levels are predictive of early recurrence in patients with bladder carcinoma. These findings suggest that serum OPG concentration may have utility as a prognostic parameter in this setting. *Cancer* 2004; 101:1794-802. © 2004 American Cancer Society.

KEYWORDS: osteoprotegerin, tumor necrosis factor–related apoptosis-inducing ligand, bladder carcinoma, prognostic marker.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its receptors participate in the cytotoxic and apoptotic mechanisms mediated by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[1,2] TRAIL is a member of the TNF family and has been shown to induce apoptosis in a variety of malignant cells, including bladder carcinoma cells.[3,4] TRAIL-induced apoptosis appears to be restricted to malignant cells, although the mechanisms underlying this selectivity are unclear.[5,6] TRAIL mediates apoptosis via two membrane-bound receptors, DR4 and DR5, both of which contain cytoplasmic death domains.[7,8] Two additional cell surface receptors, DcR1 and DcR2, also bind TRAIL; however, these receptors do not induce apoptosis and instead function as decoys.[9,10]

Recent studies have demonstrated that TRAIL also binds to osteoprotegerin (OPG) and that OPG, a member of the TNF receptor family, acts as another soluble decoy receptor for TRAIL.[11,12] Thus, OPG may compete with other TRAIL receptors on target cell surfaces and thereby inhibit TRAIL-mediated apoptosis. It is therefore conceivable that immune-mediated antitumor cytotoxicity, which relies on (among other mechanisms) the binding of TRAIL to death receptors, may be inhibited by OPG. Such inhibition may allow tumor cells to escape immune surveillance. Consequently, circulating OPG may play a critical role in the process of tumor progression.

Notably, elevated serum OPG levels have been observed in patients with several different types of hematologic and nonhematopoietic malignancies.[13,14] Previous studies have reported the prognostic significance of circulating cytotoxic lymphocytes directed against autologous tumor cells in patients with bladder carcinoma.[15] Furthermore, the prognostic significance of soluble Fas and Fas ligand levels in serum samples from patients with bladder carcinoma has been documented.[16,17] Thus, we hypothesized that serum OPG levels, like serum Fas and Fas ligand levels, may also possess prognostic significance for patients with bladder carcinoma. The current study was designed to test this hypothesis.

MATERIALS AND METHODS
Patients
Peripheral blood samples were obtained from 185 patients with initial primary bladder carcinoma who had not yet undergone surgery or received any other type of anticancer therapy. These patients included 150 men and 35 women, who ranged in age from 24 years to 89 years. Histologic diagnosis indicated that all patients had transitional cell carcinoma of the bladder. Fifty-three patients had UICC TNM (2002) Grade 1 tumors, 66 had Grade 2 tumors, and 66 had Grade 3 tumors. The TNM status distribution was as follows: Tis, $n = 10$; Ta, $n = 91$; T1, $n = 45$; T2, $n = 10$; T3, $n = 16$; and T4, $n = 4$; N1–3, $n = 5$; and M1, $n = 4$. All documented metastases occurred in the lung. Blood samples also were collected from 41 healthy donors who had no history of malignant disease. At collection, all blood samples were confirmed to be negative for findings indicative of confounding diseases or conditions. Informed consent was obtained from all study participants.

Serum was isolated via centrifugation of blood samples, and all isolated serum samples were frozen and stored at −80 °C for future enzyme-linked immunosorbent assays (ELISAs).

ELISA for OPG
A sandwich ELISA performed according to the manufacturer's protocol (Immundiagnostik, Bensheim, Germany) was used to measure serum OPG levels. All OPG concentration measurements were calibrated against titration curves that were generated using reference standards. Using this method, it was possible to ascertain serum OPG levels in excess of 0.14 pM. Repeat measurements yielded consistent results.

Patients were divided into two groups on the basis of serum OPG levels. Patients with 'high' levels had serum OPG concentrations that exceeded the median value, and patients with 'low' levels had serum OPG concentrations that were less than the median value.

Tumor Cells
Fresh tumor cells obtained from patients with bladder carcinoma were separated from surgical specimens for in vitro primary culturing, as is described elsewhere.[18,19] In brief, cell suspensions were prepared by treating finely minced tumor tissue samples with collagenase (concentration, 3 mg/mL; Sigma Chemical Co., St. Louis, MO). After being washed with RPMI-1640 medium (GIBCO Biocult, Glasgow, United Kingdom), cell suspensions were layered on discontinuous gradients consisting of 2 mL 100% Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and 2 mL 80% Ficoll-Hypaque in 15 mL plastic tubes and centrifuged at $400g$ for 30 minutes. Lymphocyte-rich mononuclear cells were collected from the 100% Ficoll-Hypaque interface, and tumor and mesothelial cells were collected from the 80% Ficoll-Hypaque interface. In some cases, cell suspensions enriched with tumor cells were contaminated with monocyte/macrophages, mesothelial cells, or lymphocytes. To prevent further contamination of host cells, we layered all cell suspensions on discontinuous gradients consisting of 2 mL each of 25%, 15%, and 10% Percoll (Amersham, Little Chalfont, United Kingdom) in complete medium in 15 mL plastic tubes and centrifuged these suspensions at $25g$ for 7 minutes at room temperature. Tumor cells that had been separated from lymphoid cells were collected from the bottom of the tube, washed, and suspended in complete medium. Morphologic examination of Wright–Giemsa-stained smears revealed that in most cases, contaminating nonmalignant cells accounted for < 5% of these tumor cell samples, which typically were > 93% viable according to the trypan blue dye exclusion test. Samples with < 5% contamination were accepted for use in the current analysis.

Tumor cells were suspended in RPMI-1640 medium supplemented with 25 mM 4-(2-hydroxyethyl)-

1-piperazineethanesulfonic acid, 2 mM L-glutamine, 1% nonessential amino acids, penicillin at a concentration of 100 units per mL, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (all from GIBCO Biocult); hereafter, this solution is referred to as *complete medium*. Tumor cells (concentration, 2 × 10^5 cells per mL) were incubated in complete medium for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2, after which the culture medium was collected and centrifuged. Supernatants then were frozen and stored at −80 °C for future ELISAs.

The human bladder carcinoma cell lines T24, J82, and HT1197 were maintained in complete medium as monolayers on plastic dishes.[20,21]

Measurement of Direct Cytotoxicity Using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The MTT assay was used to assess direct tumor cell lysis, as has been described previously.[22,23] In brief, 100 μL of an HT1197 bladder carcinoma cell suspension (2 × 10^4 cells) was added to each well of a set of 96-well flat-bottomed microtiter plates (Corning Glassworks, Corning, NY), and each plate was incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the supernatant was aspirated, tumor cells were washed 3 times with RPMI medium, and 200 μL TRAIL plus serum or culture supernatant was aliquotted into 96-well plates. Each plate was incubated for 3 days at 37 °C. Following this incubation step, 20 μL MTT working solution (concentration, 5 mg/mL; Sigma Chemical Co.) was added to each culture well, and cultures were subsequently incubated for 4 hours at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium then was removed from the microtiter wells and replaced with 100 μL isopropanol (Sigma Chemical Co.) supplemented with 0.05 N HCl. The absorbance of each well at 540 nm was measured using a microculture plate reader (Immunoreader; Japan Intermed Co., Tokyo, Japan). Percent cytotoxicity was calculated using the following formula: *% cytotoxicity* = [1 − (*absorbance of experimental wells/absorbance of control wells*)] × 100.

Statistical Analysis

All measurements were made in triplicate. Disease-specific survival rates and postoperative tumor-free intervals were calculated using the Kaplan–Meier method. The generalized Wilcoxon test and the Cox–Mantel test were used to evaluate statistical differences in survival rate and tumor-free interval between patients with high serum OPG levels and patients with low serum OPG levels. The Student *t* test and the

Pearson correlation test were used for all other statistical analyses. *P* values ≤ 0.05 were considered indicative of statistical significance.

RESULTS
Circulating OPG Levels in Serum from Healthy Individuals and in Serum from Patients with Bladder Carcinoma

ELISA was used to evaluate serum OPG levels in samples obtained from healthy control individuals (*n* = 41) and from patients with bladder carcinoma (*n* = 185). The mean serum OPG concentrations in these two groups were 4.1 and 12.9 pM, respectively (Fig. 1). Thus, OPG levels were elevated by a factor of approximately 3 in serum samples obtained from patients with bladder carcinoma. A relatively high serum OPG concentration (> 10 pM) was noted in 1 healthy control individual; however, this individual was negative for all other confounding diseases and conditions.

Serum OPG Levels in Patients with Bladder Carcinoma According to Histologic Stage and Grade

Serum OPG levels in patients with bladder carcinoma were analyzed according to histologic stage and tumor grade. Patients with muscle-invasive (T2–4N0M0) bladder carcinoma were found to have significantly higher serum OPG concentrations compared with patients who had superficial (Tis, Ta, or T1N0M0) disease (Fig. 2). Furthermore, serum OPG levels were significantly elevated in patients with T1 bladder carcinoma relative to patients with Ta disease and in patients with metastatic bladder carcinoma relative to patients with muscle-invasive disease. With regard to tumor grade, serum OPG levels were significantly higher in patients with Grade 2 bladder carcinoma compared with patients who had Grade 1 disease (Fig. 3). Likewise, serum OPG levels in patients with Grade 3 bladder carcinoma were significantly higher than those observed in patients with Grade 2 disease. These results indicate that serum OPG levels tend to increase with increasing disease stage and increasing tumor grade, a finding that is supported by preliminary experiments demonstrating that postsurgical serum OPG levels are significantly lower than serum OPG levels measured before curative surgery (data not shown).

Correlation between Serum OPG Levels and Postoperative Tumor-Free Period in Patients with Superficial (Ta or T1) Bladder Carcinoma

The postoperative clinical courses of patients with superficial (Ta or T1) bladder carcinoma who underwent transurethral resection were retrospectively evaluated. Postoperative tumor-free intervals were estimated using the Kaplan–Meier method. For the
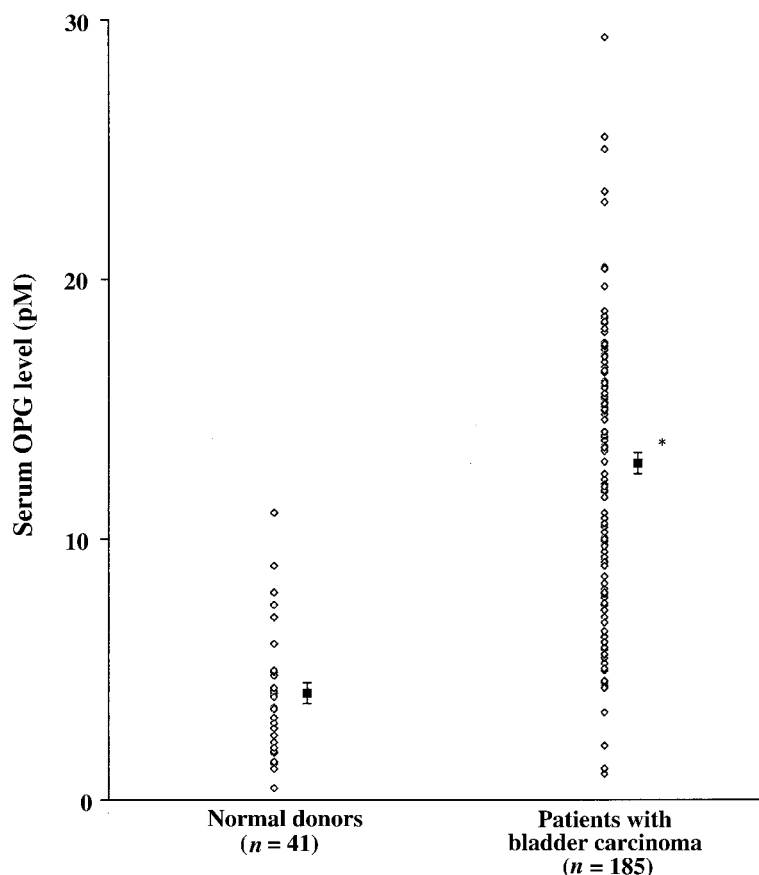
FIGURE 1. Constitutive osteoprotegerin (OPG) levels in serum samples from patients with bladder carcinoma ($n = 185$) and from healthy donors ($n = 41$). Serum OPG levels were quantified using an enzyme-linked immunosorbent assay as described in Materials and Methods. The mean serum level in patients with bladder carcinoma was approximately three times the mean level in healthy donors. Filled squares with error bars represent mean values $\pm$ standard errors. *$P < 0.05$ for comparison with healthy donors.

purposes of this subanalysis, patients with superficial bladder carcinoma were divided into two groups—those with high serum OPG levels (i.e., OPG levels greater than the median value) and those with low serum OPG levels (i.e., OPG levels less than the median value). Within the subpopulation of patients with superficial bladder carcinoma, after a follow-up period of 5 years, those who had low serum OPG levels tended to have a longer postoperative tumor-free interval compared with those who had high serum OPG levels (Fig. 4). This finding suggests that serum OPG concentration may be a significant prognostic parameter for patients with Ta or T1 bladder carcinoma.

Correlation between Serum OPG Level and Postoperative Clinical Course in Patients with Muscle-Invasive (T2–4) Bladder Carcinoma

The postoperative clinical courses of patients with muscle-invasive (T2–4N0M0) bladder carcinoma who underwent radical cystectomy also were retrospectively evaluated using the Kaplan–Meier method. In this subanalysis, again, patients were divided into two groups—those with high serum OPG levels (i.e., OPG levels greater than the median value) and those with

low serum OPG levels (i.e., OPG levels less than the median value). Among patients with muscle-invasive bladder carcinoma, the 5-year disease-specific survival rate was found to be higher for those with low serum OPG levels than for those with high serum OPG levels (Fig. 5). This finding suggests that OPG concentration may also be a significant prognostic factor for patients with muscle-invasive bladder carcinoma, with low serum OPG levels being considered a favorable prognostic indicator.

Effects of Sera Obtained from Patients with Bladder Carcinoma and of Bladder Carcinoma Cell Culture Supernatants on TRAIL-Mediated Cytotoxicity against HT1197 Cells

It has been reported elsewhere that OPG inhibits TRAIL-mediated cytotoxicity.[11,12] We attempted to investigate this inhibitory effect by assessing the extent to which serum from patients with bladder carcinoma and bladder carcinoma cell culture supernatants were able to block TRAIL-mediated cell death. Because the HT1197 bladder carcinoma cell line does not secrete OPG (data not shown), TRAIL-mediated cytotoxicity against HT1197 cells was considered strictly indicative
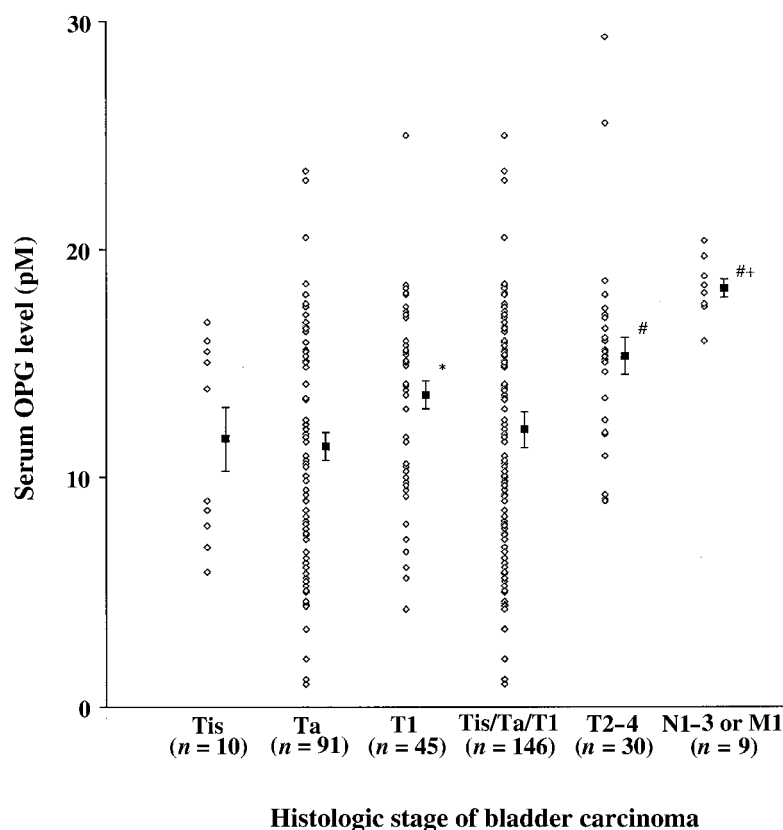
FIGURE 2. Serum osteoprotegerin (OPG) levels according to histologic stage in patients with bladder carcinoma. Serum OPG levels were quantified using an enzyme-linked immunosorbent assay as described in Materials and Methods. Filled squares with error bars represent mean values $\pm$ standard errors. $*P < 0.05$ for comparison with patients who had TaN0M0 disease. $^{\#}P < 0.05$ for comparison with patients who had TisN0M0, TaN0M0, or T1N0M0 disease. $^{+}P < 0.05$ for comparison with patients who had T2–4N0M0 disease.

of the inhibitory activity of OPG in serum samples and culture supernatants. Serum samples from patients with bladder carcinoma and bladder carcinoma cell culture supernatants both were found to significantly inhibit TRAIL-mediated cytotoxicity (Table 1). These results suggest that OPG that is present in serum and in culture supernatants (as assessed using ELISA) is biologically active and can reduce TRAIL-mediated cytotoxicity.

DISCUSSION

In the current study, it was found that serum OPG levels were higher in patients with bladder carcinoma than in healthy volunteers and that serum OPG concentration was positively correlated with disease progression and tumor grade in patients with bladder carcinoma. To our knowledge, the current study was the first to demonstrate that after a 5-year follow-up period, among patients with superficial (Ta or T1) bladder carcinoma, those with low serum OPG levels tended to have longer tumor-free intervals compared with those who had high serum OPG levels. We also found that among patients with muscle-invasive bladder carcinoma, those with low serum OPG levels had a higher 5-year disease-specific survival rate than did those with high serum OPG levels. Although we are

reporting on a limited number of patients monitored over a relatively short follow-up period, our preliminary data indicate that serum OPG levels may be a significant prognostic factor for patients with bladder carcinoma.

Several studies have measured OPG levels in serum. With regard to hematologic disorders, serum OPG levels were found to be higher in patients with Hodgkin disease and in patients with non-Hodgkin lymphoma than in healthy volunteers,[13] although that same study found no significant difference in serum OPG levels between patients with leukemia and healthy control individuals. Reduced serum OPG levels have been documented in patients with multiple myeloma.[13,24] With regard to solid tumors, serum OPG levels were found to be elevated in patients with colorectal carcinoma, patients with pancreatic carcinoma, and patients with prostate carcinoma compared with healthy individuals.[13,14] In contrast, serum OPG levels were significantly lower in patients with sarcoma compared with healthy donors.[13] Also, among patients with malignant disease, a trend toward increased serum OPG levels in patients with metastatic disease compared with patients who had localized disease was documented.[13] Likewise, the current study has demonstrated that patients with bladder carcinoma, and
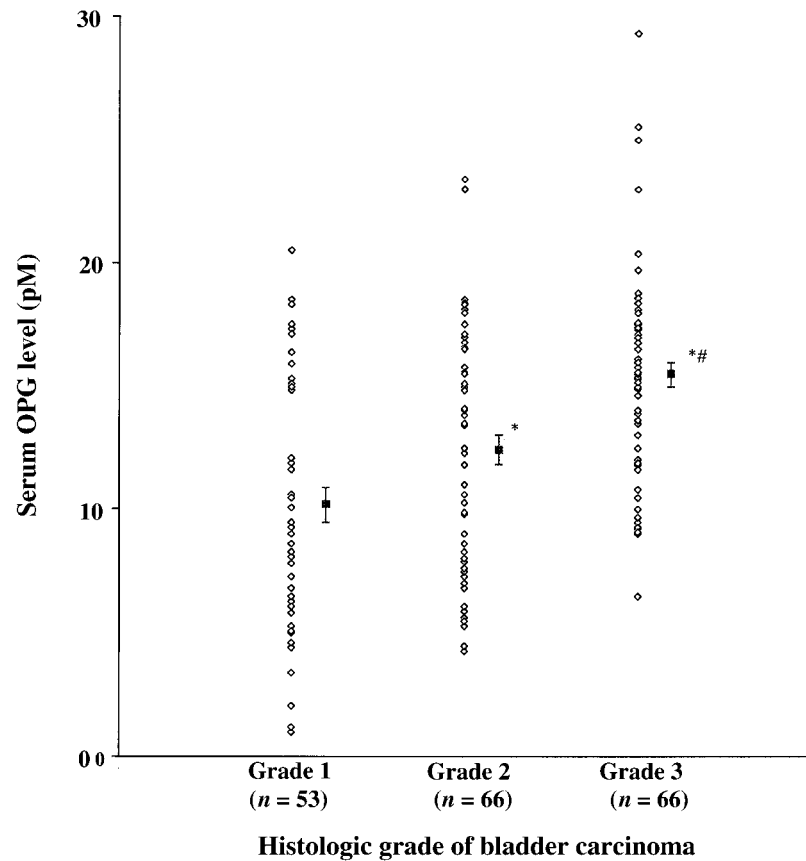
FIGURE 3. Serum osteoprotegerin (OPG) levels according to histologic grade in patients with bladder carcinoma. Serum OPG levels were quantified using an enzyme-linked immunosorbent assay as described in Materials and Methods. Filled squares with error bars represent mean values ± standard errors. *$P < 0.05$ for comparison with patients who had Grade 1 disease. #$P < 0.05$ for comparison with patients who had Grade 2 disease.
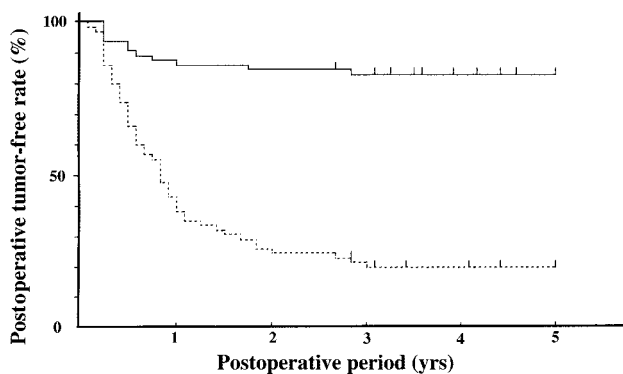
FIGURE 4. Relation between serum osteoprotegerin (OPG) concentration and postoperative tumor-free interval in patients with superficial (Ta or T1) bladder carcinoma who underwent transurethral resection. Postoperative tumor-free intervals were evaluated using the Kaplan–Meier method. Serum OPG concentrations greater than the median value were regarded as 'high' concentrations (dashed line; $n = 65$), and serum OPG concentrations less than the median value were regarded as 'low' concentrations (solid line; $n = 65$). At 5 years' follow-up, there was a significant difference between these two groups in terms of tumor-free survival duration ($P < 0.01$ [Cox–Mantel test and generalized Wilcoxon test]).
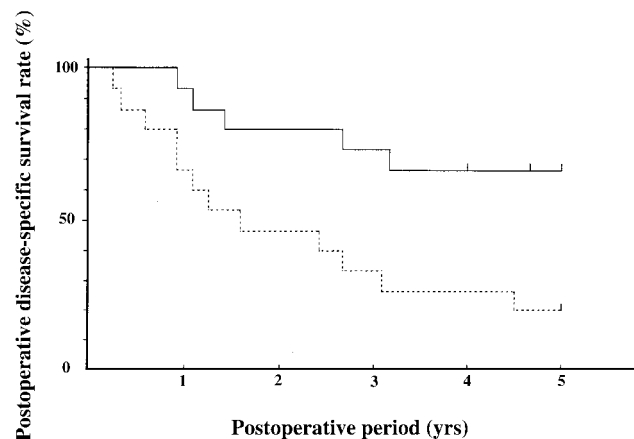
FIGURE 5. Relation between serum osteoprotegerin (OPG) levels and disease-specific survival in patients with muscle-invasive (T2–4N0M0) bladder carcinoma who underwent radical cystectomy. Postoperative clinical course was assessed using the Kaplan–Meier method. Serum OPG concentrations greater than the median value were regarded as 'high' concentrations (dashed line; $n = 15$), and serum OPG concentrations less than the median value were regarded as 'low' concentrations (solid line; $n = 15$). At 5 years' follow-up, there was a significant difference between these two groups in terms of the disease-specific survival rate ($P < 0.05$ [Cox–Mantel test and generalized Wilcoxon test]).

TABLE 1
Inhibition of TRAIL-Mediated Cytotoxicity by Serum Samples from Patients with Bladder Carcinoma and Bladder Carcinoma Cell Culture Supernatants

Serum sample/supernatant source	TNM status	Tumor grade	OPG concentration (pM)	Mean % cytotoxicity against HT1197 bladder carcinoma cells ± SD[a]
TRAIL alone (10 pM)	—	—	—	24.3 ± 2.4
Patient 1	TaN0M0	1	20.5	10.4 ± 0.3[b]
Patient 2	T2N0M0	3	29.3	8.0 ± 0.2[b]
Patient 3	TaN0M0	3	23.0	14.6 ± 0.3[b]
Patient 4	T3N0M0	3	25.0	8.7 ± 0.1[b]
Patient 5	TaN0M0	2	23.0	13.0 ± 0.1[b]
Patient 6	T1N0M0	2	74.0	7.7 ± 1.5[b]
Patient 7	T3N0M0	3	520.0	3.7 ± 0.9[b]
T24 cell culture	—	—	35.4	8.9 ± 1.8[b]
J82 cell culture	—	—	268.2	6.2 ± 1.5[b]

TRAIL: tumor necrosis factor–related apoptosis-inducing ligand; OPG: osteoprotegerin; SD: standard deviation.

[a] All cytotoxicity measurements were performed in triplicate.

[b] $P < 0.05$ for comparison with cytotoxicity of sample containing TRAIL only.

especially those with metastatic disease, have elevated serum OPG levels compared with healthy control individuals. Taken together, these findings suggest that serum OPG concentration may be a measure of tumor burden in patients with nonhematopoietic malignancies.

Cell-mediated immunity plays an important role in the process of immune surveillance of bladder carcinoma cells.[15,25] An important parameter of cell-mediated cytotoxicity, the activity of peripheral blood lymphocytes against autologous tumor cells is a significant and independent prognostic indicator for patients with bladder carcinoma.[15] Cytotoxic activity against autologous tumor cells is mediated by CTLs and NK cells. Furthermore, TRAIL-induced apoptosis is involved in both CTL-mediated and NK-mediated antitumor cytotoxic mechanisms,[1,2] and OPG has been implicated in the inhibition of TRAIL-mediated apoptosis.[11,12] Thus, elevated levels of OPG in the circulation may reduce cytotoxic activity against autologous tumor cells by inhibiting TRAIL-mediated apoptosis. The current study tested this hypothesis and revealed that serum samples from patients with bladder carcinoma and bladder carcinoma cell culture supernatants, all of which were high in OPG content, inhibited TRAIL-mediated cytotoxicity. This finding indicates that elevated serum OPG levels may be associated with poor prognosis as a result of OPG's inhibitory effect on TRAIL-mediated cytotoxicity. It therefore is reasonable to conclude that elevated serum OPG levels are involved in a novel mechanism by which tumor cells can escape from immune surveillance, with this mechanism promoting tumor progression in patients with bladder carcinoma.

Several features that are potentially associated with mechanisms of resistance to TRAIL-mediated apoptosis have been identified, including reduced expression of TRAIL receptors DR4 and DR5 and enhanced expression of the antagonistic TRAIL receptors DcR1 and DcR2.[7–10] The existence of multiple TRAIL receptors suggests an unexpected level of complexity in the regulation of TRAIL-mediated signaling. Antiapoptotic molecules such as Bcl-2 and Bcl-xL may be specifically associated with resistance to TRAIL-mediated apoptosis (among other forms of apoptosis).[26,27] Because TRAIL induces apoptosis in malignant cells in a caspase-dependent manner, resistance to TRAIL-mediated apoptosis may also be dependent on caspase expression levels.[28,29] FLICE-like inhibitory protein has been shown to bind to caspase-8 and prevent the activation of downstream events leading to apoptosis, including TRAIL-mediated apoptosis.[30,31] The current study suggests that OPG production by bladder carcinoma cells may represent another mechanism of resistance to TRAIL-mediated apoptosis. Nonetheless, further studies are required to elucidate the mechanisms by which bladder carcinoma cells acquire resistance to TRAIL-mediated cytotoxicity.

The precise cellular source of OPG has not been elucidated. We speculate that OPG may be derived from malignant cells and/or normal tissue. Previous reports have demonstrated that OPG is produced by prostate carcinoma cells and Hodgkin lymphoma cells.[32,33] Normal tissues, including lung, heart, liver, stomach, intestinal, kidney, skin, brain, spinal cord, thyroid gland, and bone tissue, also have been found to produce OPG.[34,35] In the current study, preliminary

experiments revealed that OPG was present in bladder carcinoma cell culture supernatants and also in the supernatants of primary cultures derived from surgical specimens. These findings suggest that both malignant cells and normal tissue produce OPG, although further studies are required to conclusively identify the cellular source of OPG in patients with bladder carcinoma.

Osteoclast differentiation recently was shown to be positively and negatively regulated by a complex signaling system involving receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and OPG. Among the key interactions that these molecules take part in are the binding of RANK on osteoclast progenitor cell surfaces to RANKL on osteoblasts during direct cell contact, an interaction that induces osteoclastogenesis and activates bone resorption, and the binding of soluble OPG to RANKL, an interaction that suppresses osteoclastogenesis by interfering with the interaction between RANK and RANKL.[36] Such suppression of osteoclastogenesis has been observed at OPG concentrations of 10–100 ng/mL.[34,37] Although some patients with bladder carcinoma have significantly elevated levels of circulating OPG, these levels are not sufficient to suppress osteoclast formation.

The current study demonstrated that serum OPG concentration may have clinical usefulness as a prognostic marker for patients with bladder carcinoma; however, only a limited number of patients with T4, N1, or M1 disease were included in the current cohort, and as a result, broad generalization of the prognostic value of serum OPG levels was not possible. Thus, our findings warrant further investigation and require validation in larger patient populations.

Although overall rates of response to chemotherapy for patients with bladder carcinoma have improved, metastasis and disease recurrence remain major problems. Therefore, novel therapeutic approaches are required for patients with metastatic or recurrent disease. The up-regulation of serum OPG levels in patients with bladder carcinoma (and especially in patients with advanced-stage or high-grade disease) compared with healthy donors suggests that OPG may be a useful molecular target for anticancer therapy. Accordingly, inhibition of the production or biologic activity of OPG may prevent the progression of bladder carcinoma. Furthermore, strategies aimed at inhibiting OPG production may increase the susceptibility of malignant cells to TRAIL-mediated killing by cytotoxic lymphocytes and thus improve clinical outcomes.

In conclusion, the data presented in the current report demonstrate that serum OPG concentration is positively correlated with histologic disease stage and tumor grade in patients with bladder carcinoma and that elevated serum OPG levels are associated with early recurrence in such patients. The observed correlation with postoperative prognosis suggests that serum OPG levels may possess utility as a prognostic marker for patients with bladder carcinoma, and in turn, accurate assessment of prognosis may aid in the selection of patients to receive intensive surgical or chemotherapeutic treatment.

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Inhibition of the Raf–MEK1/2–ERK1/2 Signaling Pathway, Bcl-$_{xL}$ Down-Regulation, and Chemosensitization of Non-Hodgkin's Lymphoma B Cells by Rituximab

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ABSTRACT

Rituximab (Rituxan, IDEC-C2B8) has been shown to sensitize non-Hodgkin's lymphoma (NHL) cell lines to chemotherapeutic drug-induced apoptosis. Rituximab treatment of Bcl-2–deficient Ramos cells and Bcl-2–expressing Daudi cells selectively decreases Bcl-$_{xL}$ expression and sensitizes the cells to paclitaxel-induced apoptosis. This study delineates the signaling pathway involved in rituximab-mediated Bcl-$_{xL}$ down-regulation in Ramos and Daudi NHL B cells. We hypothesized that rituximab may interfere with the extracellular signal-regulated kinase (ERK) 1/2 pathway, leading to decreased Bcl-$_{xL}$ expression. Rituximab (20 μg/mL) inhibited the kinase activity of mitogen-activated protein kinase kinase (MEK) 1/2 and reduced the phosphorylation of the components of the ERK1/2 pathway (Raf-1, MEK1/2, and ERK1/2) and decreased activator protein-1 DNA binding activity and Bcl-$_{xL}$ gene expression. These events occurred with similar kinetics and were observed 3 to 6 hours after rituximab treatment. Rituximab-mediated effects were corroborated by using specific inhibitors of the ERK1/2 pathway, which also reduced Bcl-$_{xL}$ levels and sensitized the NHL B cells to paclitaxel-induced apoptosis. Previous findings implicated a negative regulatory role of the Raf-1 kinase inhibitor protein (RKIP) on the ERK1/2 pathway. Rituximab treatment of NHL B cells significantly up-regulated RKIP expression, thus interrupting the ERK1/2 signaling pathway through the physical association between Raf-1 and RKIP, which was concomitant with Bcl-$_{xL}$ down-regulation. These novel findings reveal a signaling pathway triggered by rituximab, whereby rituximab-mediated up-regulation of RKIP adversely regulates the activity of the ERK1/2 pathway, Bcl-$_{xL}$ expression, and subsequent chemosensitization of drug-refractory NHL B cells. The significance of these findings is discussed.

INTRODUCTION

The B-cell lineage restricted marker CD20 is expressed on mature B cells with minimal expression on early pre-B cells and normal plasma cells. Approximately 80% to 85% of non-Hodgkin's lymphomas (NHLs) are B-cell malignancies in origin, and >95% of these express surface CD20. CD20 is neither shed from the cell surface (1) nor modulated or internalized on antibody (Ab) binding (2), which makes it a suitable target for immunotherapy. The chimeric mouse antihuman CD20 monoclonal Ab (mAb) rituximab [Rituxan, IDEC-C2B8 (3)] has significant antitumor activity and, alone or in combination with chemotherapy, has been successfully used in the treatment of patients with follicular or low-grade lymphoma (4) and aggressive diffuse large B-cell lymphoma in elderly patients (5). Treatment of CD20$^+$ NHL B cells with rituximab triggers multiple cell-damaging

mechanisms. Possible antitumor mechanisms involve Ab-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and induction of apoptosis (6).

The *in vivo* effectiveness of the combination of rituximab and drugs in the treatment of drug-resistant tumors suggests that rituximab can modify the drug-resistant phenotype by interfering with signaling pathways. In fact, we have reported that rituximab interferes with the intracellular signal transduction pathways and sensitizes NHL B-cell lines to drugs via selective down-regulation of transcription of the antiapoptotic gene products Bcl-2 [in AIDS-related NHL (ARL); ref. 7] or Bcl-$_{xL}$ (in non-ARL; ref. 8). These proteins exert their protective effects mainly in the membrane of mitochondria, where they prevent loss of membrane potential, cytochrome *c* efflux on apoptotic stimuli, and the initiation of apoptosis (9). Bcl-2 and the long alternatively spliced variant of the Bcl-x gene, Bcl-$_{xL}$, are predominantly expressed in lymphomas (10) and protect the cells from apoptosis induced by drugs, thus conferring a multidrug-resistant phenotype (9, 11, 12).

Activation of the extracellular signal-regulated kinase (ERK) 1/2 pathway by fibroblast growth factor-2 rescues small-cell lung carcinoma cells from apoptosis induced by etoposide, via up-regulation of Bcl-2 and Bcl-$_{xL}$ (13). Thus, we hypothesized that rituximab may inhibit the constitutive activity of the ERK1/2 pathway, leading to inhibition of Bcl-$_{xL}$ transcription. A negative regulatory role for the Raf-1 kinase inhibitor protein (RKIP) on the ERK1/2 pathway is described (14, 15). RKIP exerts its suppressive effects via physical association with Raf-1, thus rendering it incapable of relaying the signal to downstream molecules. Overexpression of RKIP significantly reduces the transformation efficiency of Raf-1 kinase domain BXB and decreases activator protein (AP)-1–dependent transcription (14, 15). Thus, we further hypothesized that rituximab may up-regulate RKIP expression, resulting in inhibition of the ERK1/2 pathway, diminished Bcl-$_{xL}$ expression, and chemosensitization of NHL B cells.

This study tested both of the above hypotheses using the Bcl-2$^-$/Bcl-$_{xL}$ Ramos and the Bcl-2$^+$/Bcl-$_{xL}^+$ Daudi NHL B-cell lines, and the following questions were investigated: (*a*) whether rituximab inhibits the ERK1/2 pathway; (*b*) whether rituximab decreases AP-1 DNA binding activity; (*c*) whether pharmacological interruption of the ERK1/2 pathway mimics rituximab-mediated effects such as inhibition of Bcl-$_{xL}$ expression, chemosensitization, and inhibition of proliferation; and (*d*) whether rituximab up-regulates RKIP expression and potentiates its association with Raf-1, thus interfering with the activity of the ERK 1/2 signaling pathway.

MATERIALS AND METHODS

Tumor Cell Lines

The CD20$^+$ human B-cell lines Daudi and Ramos (American Type Culture Collection, Manassas, VA) were maintained in sterile 75-cm^2 tissue culture flasks in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (to ensure the absence of complement) as described previously (7, 8). The pEBB-puro-Bcl-x-HA construct was generated by polymerase chain reaction (PCR) cloning of human Bcl-x, which then was inserted into the *Bam*HI and *Not*I sites of pEBB-puro-HA in-frame with the 3′ influenza

hemagglutinin (HA) tag (16). The cells were then pulsed using electroporation

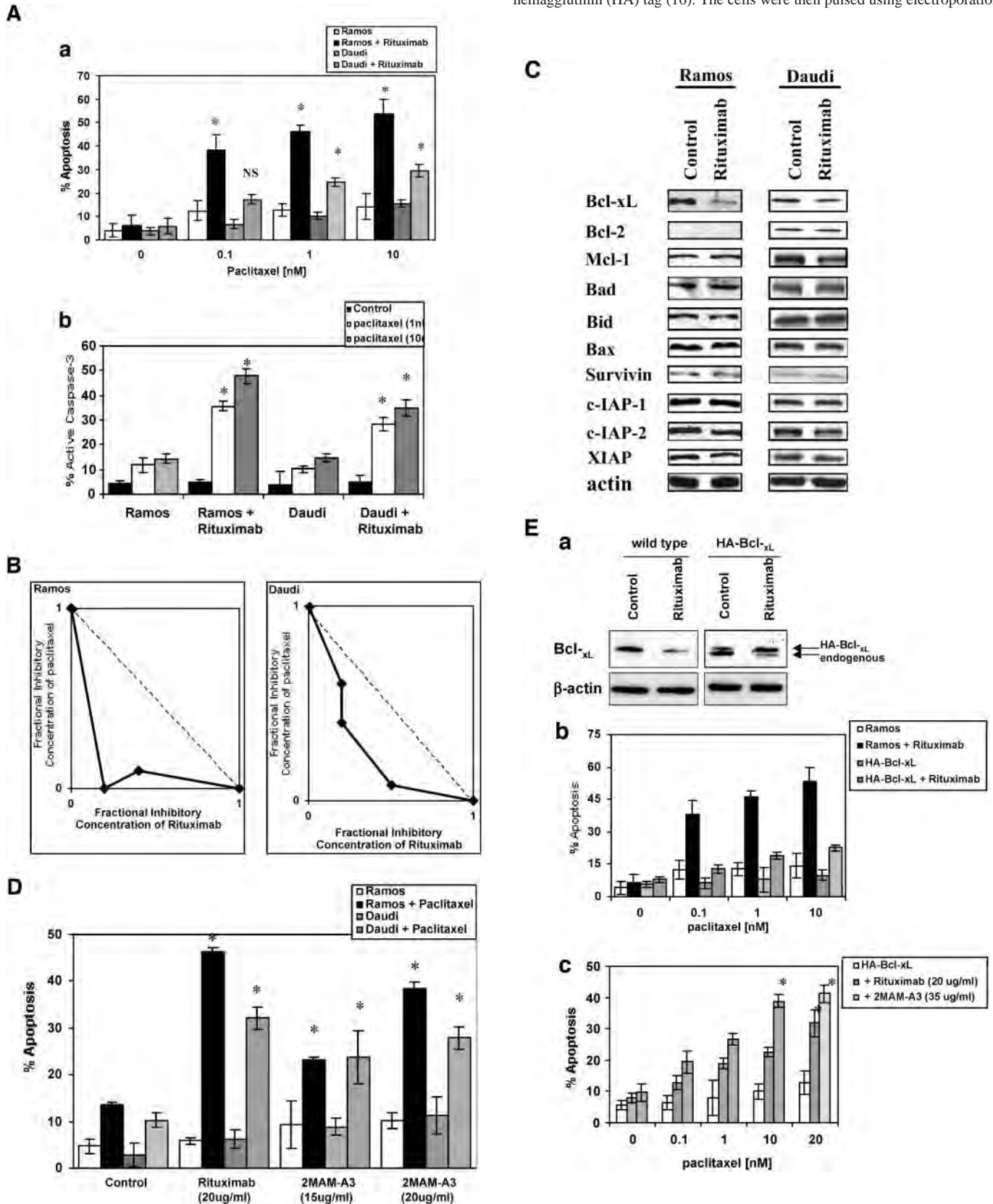Fig. 1. Rituximab-mediated effects, alone and in combination with paclitaxel, on the NHL B-cell lines. *A,* Ramos and Daudi cells were either left untreated (control) or pretreated with rituximab (20 μg/mL, 24 hours). Thereafter, the cells were washed, fresh medium was added, and the cells were incubated with various concentrations of paclitaxel (0.1, 1, and 10 nmol/L, 18 hours; ref. 8). The cells were then stained with PI solution, and cell cycle analysis was assessed by flow cytometry. The percentage of apoptosis is represented as the percentage of tumor cells with hypodiploid DNA accumulating at the sub-G_0 phase of the cell cycle. An aliquot of the same samples was stained with anti-active caspase-3 mAb to validate the PI procedure (24). *B,* Synergy in apoptosis is achieved by the combination of rituximab and paclitaxel as determined by isobolographic analysis (26). *C,* examination of a panel of pro- and antiapoptotic proteins after exposure to rituximab. Tumor cells were either left untreated (control) or treated with rituximab (20 μg/mL, 24 hours), and total cell lysates (40 μg) were subjected to immunoblot analysis. *D,* Tumor cells were left untreated or treated with rituximab (20 μg/mL, 24 hours) or 2MAM-A3 (15 and 20 μg/mL,

at 250 V/975 μF and then selected and maintained in 2.5 μg/mL puromycin. This cell line was provided by Dr. G. Cheng (University of California at Los Angeles, Los Angeles, CA). Tumor cell cultures were incubated in a controlled atmosphere incubator at 37°C with saturated humidity and an atmosphere of 95% air and 5% CO_2 at a density of 0.5×10^6 cells/mL.

Reagents

A stock solution of paclitaxel [6 mg/mL in dimethyl sulfoxide; Bristol Myers Squibb (New York, NY)] was kept at room temperature. For each experiment, paclitaxel was diluted with medium to obtain the indicated concentrations. The dimethyl sulfoxide concentration did not exceed 0.1% in any experiment. Rituximab (stock, 10 mg/mL) was obtained commercially.

Mouse anti–Bcl-$_{xL}$, anti-Mcl-1, anti–phospho-p38 (Tyr^{182}), anti–c-Jun, and anti–c-Fos mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti–Bcl-2 mAb was purchased from DAKO (Carpinteria, CA). Rabbit anti-Bad, anti-Bid, and anti-Bax polyclonal Abs were purchased from Cell Signaling (Beverly, MA). Rabbit anti-survivin, anti–c-IAP-1, anti–c-IAP-2, and anti-XIAP polyclonal Abs were purchased from Proscience (Poway, CA) and Trevegen (Gaithersburg, MD), respectively. Mouse anti-actin mAb was purchased from Chemicon (Temecula, CA). Rabbit anti-RKIP (NH$_2$-terminal) polyclonal Ab was purchased from Zymed (San Francisco, CA). Rabbit anti–phospho-mitogen-activated protein kinase kinase (MEK) 1/2 (Ser^{222}), anti-MEK1/2, anti–c-Raf-1, anti–phospho-c-Raf-1 (Tyr$^{340/341}$), anti-ERK1/2, anti–phospho-ERK1/2 (Thr^{185}/Tyr^{187}), anti–phospho-c-Jun NH$_2$-terminal kinase (JNK) 1/2 (Thr^{183}/Tyr^{185}) polyclonal Abs; the mitogen-activated protein kinase (MAPK) kinase substrate 4; and PD098059 (17) were obtained from Biosource (Camarillo, CA). U0126 (18) was purchased from Promega (Madison, WI). 2-Methoxyantimycin-A3 [2MAM-A3 (19)] and GW5074 (20) were purchased from Biomol (Plymouth, PA) and Tocris Cookson Inc. (Ellisville, MO), respectively.

Immunoblotting Analysis for Protein Expression

This was performed as described previously (7, 8, 21).

Immunoprecipitation of Raf-1 Kinase Inhibitor Protein

Cells (± rituximab) were harvested and pelleted at $14,000 \times g$ for 2 minutes. The pellets were then resuspended and dissolved in 500 μL of ice-cold radioimmunoprecipitation assay buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2 μg of rabbit anti–Raf-1 Ab and subsequently incubated with 30 μL of Immuno-Pure Plus Immobilized protein A beads (ref. 22; Pierce, Rockford, IL) for 4 hours at 4°C on a shaking platform. After centrifugation for 1 minute at $14,000 \times g$, the supernatant was discarded, and the immunoprecipitates were washed twice with 1.0 mL of ice cold radioimmunoprecipitation assay buffer before assay. RKIP was immunoprecipitated from the lysate by use of a rabbit polyclonal Ab (1:2,000 dilution). The immunoprecipitate was resolved on a 10% SDS-PAGE gel and visualized by autoradiography.

Kinase Assay

Alterations in the kinase activity of MEK1/2 on rituximab treatment were assessed by the ability of MEK1/2 to phosphorylate the MAPK substrate 4 (ERK1/2; using ERK1/2 peptide containing residues 172–192 (N-ADPDH-DHTGFLTEYVATRWRR-C) as substrate], using a slightly modified version of previous methods (17, 18).

Assessment of Apoptosis by Flow Cytometric Analysis

DNA Fragmentation Assay. The percentage of apoptotic cells was determined by evaluation of propidium iodide (PI)-stained preparations (23) of tumor cells treated under various conditions as reported previously (8). The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at the sub-G$_0$ phase of the cell cycle.

Evaluation of Active Caspase-3 Levels. To validate the PI staining technique for the measurement of apoptosis, levels of active caspase-3 were evaluated as described previously (24).

Electrophoretic Mobility Shift Assay

After treatment with rituximab and inhibitors, cells (10^6 cells per treatment) were withdrawn as a function of time, pelleted, and washed twice with ice-cold PBS. After washing, cells were lysed in 1.0 mL of Nonidet P-40 lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L MgCl$_2$, and 0.5% Nonidet P-40] on ice for 5 minutes. Samples were centrifuged at $2,000 \times g$ at 4°C for 5 minutes to pellet the nuclei, and supernatants were subsequently removed. Nuclei were washed once in Nonidet P-40 buffer and twice in ice-cold PBS. Nuclei were then lysed in nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L MgCl$_2$, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L dithiothreitol] and sonicated at 4°C for 30 seconds. A detergent-compatible protein assay kit was used to determine the protein concentration of the nuclear extracts. Nuclear proteins were mixed for 30 minutes at room temperature with biotin-labeled oligonucleotide AP-1 probe (5′-CGCTTGATGACTCAGCCG-GAA-3′; ref. 25) using the electrophoretic mobility shift assay (EMSA) kit purchased from Panomics Inc. (Redwood City, CA) according to the manufacturer's instructions, as described previously (24). For the supershift assays, 1 μL of the appropriate Abs was added to the nuclear extracts for 20 minutes on ice before the addition of the labeled probe.

Reverse Transcription-Polymerase Chain Reaction

The reverse transcription-PCR analysis was performed as described previously (21, 24). After reverse transcription, 2.5 μL of cDNA were amplified using the following Bcl-$_{xL}$ gene-specific primers: forward, 5′-ACCATGTCT-CAGAGCAACCGGGAGCT-3′; and reverse, 5′-TCATTTCCGACTGAA-GAGTGAGCC-3′. Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific glyceraldehyde-3-phosphate dehydrogenase primers: forward, 5′-GAACATCATCCCTGCCTCTACTG-3′; and reverse, 5′-GT TGCTGT AGCCAAATTCGTTG-3′.

Luciferase Bcl-x Promoter Reporter Assay

A 650-bp region of Bcl-x promoter spanning −640 to +9 relative to the transcription start site was inserted between the *Xho*I and *Hind*III sites of the pGL2-Basic luciferase reporter vector to generate the Bcl-x wild-type (WT) promoter as described previously (16). Cells were then transfected by electroporation using pulse at 270 V/975 μF with 10 μg of Bcl-x WT promoter or empty plasmid. After transfection, the cells were cultured in 12-well plates and allowed to recover for 36 hours. The cells were then either left untreated or treated with PD098059 (20 μg/mL) or rituximab (20 μg/mL) for 18 hours. Cells were then harvested in $1\times$ lysis buffer, and luciferase activity was measured according to the manufacturer's instructions (Promega, Pittsburgh, PA).

XTT Proliferation Assay

Inhibition of proliferation was assessed using the standard 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) assay kit (Roche, Indianapolis, IN), which measures the metabolic activity of viable cells (27). The percentage of proliferation was calculated using the background-corrected reading as follows: Proliferation (%) = [(absorbance of sample wells/absorbance of untreated cells)] × 100.

Isobolographic Analysis for Determination of Synergy

Determination of the synergistic *versus* additive *versus* antagonistic cytotoxic effects of the combination treatment of the tumor cells by rituximab and paclitaxel was assessed by isobolographic analysis as described previously (26).

Statistical Analysis

Assays were set up in triplicates, and the results were expressed as the mean ± SD. Statistical analysis and P value determinations were done by

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7 hours) followed by paclitaxel treatment (10 nmol/L, 18 hours) and analyzed for the percentage of apoptosis. *E,* Bcl-$_{xL}$–overexpressing cells were treated under the conditions described in *A−D* and analyzed by Western blot and flow cytometry. Results represent the mean ± SD of duplicate samples from three independent experiments. *, $P < 0.05$ (significant); *NS*, not significant, compared with paclitaxel treatment alone.

two-tailed paired t test with a confidence interval of 95% for determination of the significance of differences between the treatment groups. $P < 0.05$ was considered to be significant. Analysis of the variance was used to test the significance among the groups. InStat 2.01 software was used for analysis.

RESULTS

Rituximab Sensitizes the Ramos and Daudi NHL B Cells to Paclitaxel-Induced Apoptosis by Down-Regulation of Bcl-$_{xL}$ Expression

Optimal concentration of rituximab (20 μg/mL) (28) sensitizes the CD20$^+$ (>95%) Ramos and Daudi cells to apoptosis induced by clinically achievable subtoxic concentrations of paclitaxel (8, 29). A close correlation between PI$^+$ cells and those possessing active caspase-3 was established (ref. 24; Fig. 1A). Rituximab alone did not induce significant apoptosis beyond the background levels in both cell lines. All three concentrations of paclitaxel induced significant apoptosis in rituximab-pretreated Ramos and Daudi cells compared with the untreated control cells (Fig. 1A). The observed augmentation of apoptosis by the combination treatment of rituximab and paclitaxel was synergistic as determined by isobolographic analysis (Fig. 1B).

Western blot analysis of the total cell lysates (40 μg) of tumor cells [± rituximab (20 μg/mL), 24 hours] showed that in Bcl-2–deficient Ramos (30) and Bcl-2–expressing Daudi cells, rituximab selectively decreased the expression of Bcl-$_{xL}$ but had no effect on the expression of several other apoptosis-associated proteins tested (Fig. 1C). To confirm the protective role of Bcl-$_{xL}$ in paclitaxel-induced apoptosis, 2MAM-A3, which specifically impairs the function of Bcl-$_{xL}$ (19), was used. The cells were grown either in complete medium (control) or in complete medium supplemented with various concentrations of 2MAM-A3 (15 and 20 μg/mL, 7 hours) followed by treatment paclitaxel (10 nmol/L, 18 hours). As shown, 2MAM-A3 by itself was inefficient in killing the tumor cells, but it was capable of significantly augmenting paclitaxel-induced apoptosis in both cell lines, albeit to varying degrees (Fig. 1D).

To ascertain the protective role of Bcl-$_{xL}$ against drug-induced apoptosis, Ramos cells were stably transfected with a HA-tagged Bcl-$_{xL}$–expressing construct (16). These cells expressed higher levels of Bcl-$_{xL}$ compared with the parental cell line (Fig. 1E, a) and exhibited higher resistance to paclitaxel [and cisplatin, etoposide, and Adriamycin (data not shown)] compared with the parental cell line. Rituximab only reduced the levels of endogenous Bcl-$_{xL}$ in these cells but not the ectopic Bcl-$_{xL}$ driven by the overexpressing vector (Fig. 1E, a), and rituximab was not as efficient in sensitizing these cells to paclitaxel compared with the parental cells (Fig. 1E, b). However, higher concentrations of 2MAM-A3 (35 μg/mL) than those used for the parental cells (15 and 20 μg/mL; Fig. 1D) sensitized the HA-Bcl-$_{xL}$ cells to paclitaxel (Fig. 1E, c).

These results demonstrate that Bcl-$_{xL}$ regulates the resistant phenotype and that rituximab, through down-regulation of Bcl-$_{xL}$, sensitizes the Ramos and Daudi cells to paclitaxel-induced apoptosis in a synergistic manner.

Rituximab Diminishes the Constitutive Activity of the ERK1/2 MAPK Signaling Pathway in Ramos and Daudi NHL B-Cell Lines

The ERK1/2 MAPK signaling pathway regulates the transcriptional expression of Bcl-$_{xL}$ (31, 32). Total cell lysates of Ramos and Daudi cells treated with rituximab (1–24 hours) were subjected to immunoblotting using phospho-specific and non–phospho-specific Abs for proteins in the ERK1/2 pathway. A slight increase in the phospho-dependent state of Raf-1 and MEK1/2 was observed 1 hour after

rituximab treatment. After this transient period, however, rituximab decreased the phospho-dependent state of Raf-1, MEK1/2, and ERK1/2 in a time-dependent manner beginning 3–6 hours after treatment, which was maintained up to 24 hours. The basal level (phospho-independent state) of these proteins remained unaltered during the entire (24-hour) experiment (Fig. 2A). The temporal expression of Bcl-$_{xL}$, which is regulated in part by the ERK1/2 pathway (33, 34), was also examined. Rituximab decreased the expression of Bcl-$_{xL}$ at the protein level as a function of time starting between 3 and 6 hours after treatment; the decrease was more pronounced at later time points (Fig. 2A).

In contrast to our findings with monomeric rituximab, previous findings demonstrated that cross-linking rituximab induces apoptosis and activates the MAPK pathways in NHL and B-cell chronic lymphocytic leukemia cells (33, 34). To validate these observations, cross-linked rituximab was generated by using the secondary antihuman immunoglobulin (50 μg/mL) in combination with rituximab. Cross-linking rituximab, but not monomeric rituximab, resulted in robust and sustained activation of ERK1/2 and p38 (and to a lesser extent, JNK1/2; Fig. 2B) but had no effect on the basal levels of these proteins (data not shown). Activation of the MAPK pathways was accompanied by a substantial induction of apoptosis in both cell lines at later time points (18–24 hours; data not shown).

To ascertain whether the observed dephosphorylation also resulted in decreased kinase activity of the ERK1/2 pathway, an *in vitro* (in gel) kinase assay was performed. The MEK1/2 kinase activity (using ERK1/2 peptide containing residues 172–192 as substrate) of tumor cells (±20 μg/mL rituximab) was assessed. Rituximab decreased MEK1/2 kinase activity as shown by the inability of rituximab-treated cells to phosphorylate the ERK1/2 peptide (Fig. 2C), which was reversed on treatment with cross-linked rituximab (data not shown).

Collectively, these results demonstrate the ability of rituximab to negatively regulate the activity of the ERK1/2 signaling pathway and concomitantly decrease the protein level of Bcl-$_{xL}$. These results also show that the molecular signaling pathways triggered by monomeric *versus* cross-linked rituximab are different.

Pharmacological Inhibition of the ERK1/2 MAPK Signaling Pathway Mimics the Effects of Rituximab

Sensitization to Paclitaxel-Induced Apoptosis. The involvement of the ERK1/2 signaling pathway in the resistance of Ramos and Daudi cells to paclitaxel and inhibition of this pathway by rituximab suggest that specific inhibition of this pathway should mimic rituximab. PD098059 exerts its effects by binding to the inactive form of MEK1/2 and prevents MEK1/2 activation by Raf-1, thus inhibiting the activation of ERK1/2 (17). U0126 blocks the phosphorylation and activation of ERK1/2 (18), and GW5074 is a specific inhibitor of Raf-1 (20). Optimal concentrations of the inhibitors were determined by pilot studies and are in accordance with previous reports (17, 18, 20). The cells were pretreated with the inhibitors (GW5074: 10 μmol/L, 45 minutes; PD098059: 20 μg/mL, 45 minutes; U0126: 10 μmol/L, 45 minutes) followed by paclitaxel (10 nmol/L, 18 hours) treatment. Paclitaxel (10 nmol/L) induced modest apoptosis, whereas the inhibitors were nontoxic to the cells. However, the inhibitors sensitized the cells to paclitaxel (Fig. 3A).

To determine the specificity of the inhibitors, tumor cells were treated with inhibitors under the conditions described above. Total cell lysates were analyzed by immunoblotting using specific Abs for pERK1/2, p-p38, and pJNK1/2 MAPKs. The inhibitors specifically blocked ERK1/2 phosphorylation but had no effect on p38 and JNK1/2 phosphorylation. 2MAM-A3 had no modulatory effect on pERK1/2, p-p38, or pJNK1/2 (Fig. 3B). The phospho-independent
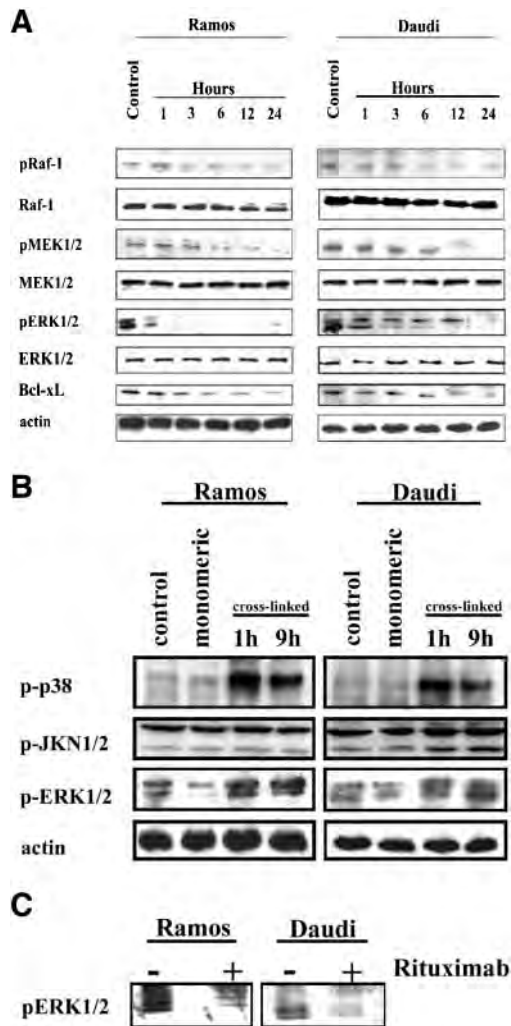
Fig. 2. Rituximab inhibits the ERK1/2 signaling pathway. *A*, dephosphorylation of the components of the ERK1/2 pathway by monomeric rituximab. *B*, activation of the MAPK pathways by cross-linked rituximab. *C*, inhibition of MEK1/2 kinase activity by monomeric rituximab. Ramos and Daudi cells were grown in complete medium in the absence (control) or presence of rituximab (20 μg/mL) for various time points (1, 3, 6, 12, and 24 hours) or cross-linked rituximab (20 μg/mL rituximab +50 μg/mL antihuman immunoglobulin). Total cell lysates (40 μg) were subjected to Western blot analysis using phospho-specific and non–phospho-specific Abs for various components of the ERK1/2, p38, and JNK1/2 MAPK pathways (*A* and *B*) or kinase assay (*C*). The results are representative of three independent experiments.

Regulation of Bcl-xL Expression by the ERK1/2 Pathway

It has been reported that activation of the ERK1/2 pathway leads to transcriptional activation of AP-1 and AP-1–dependent Bcl-xL gene expression (31, 32). Rituximab decreased the activation of the
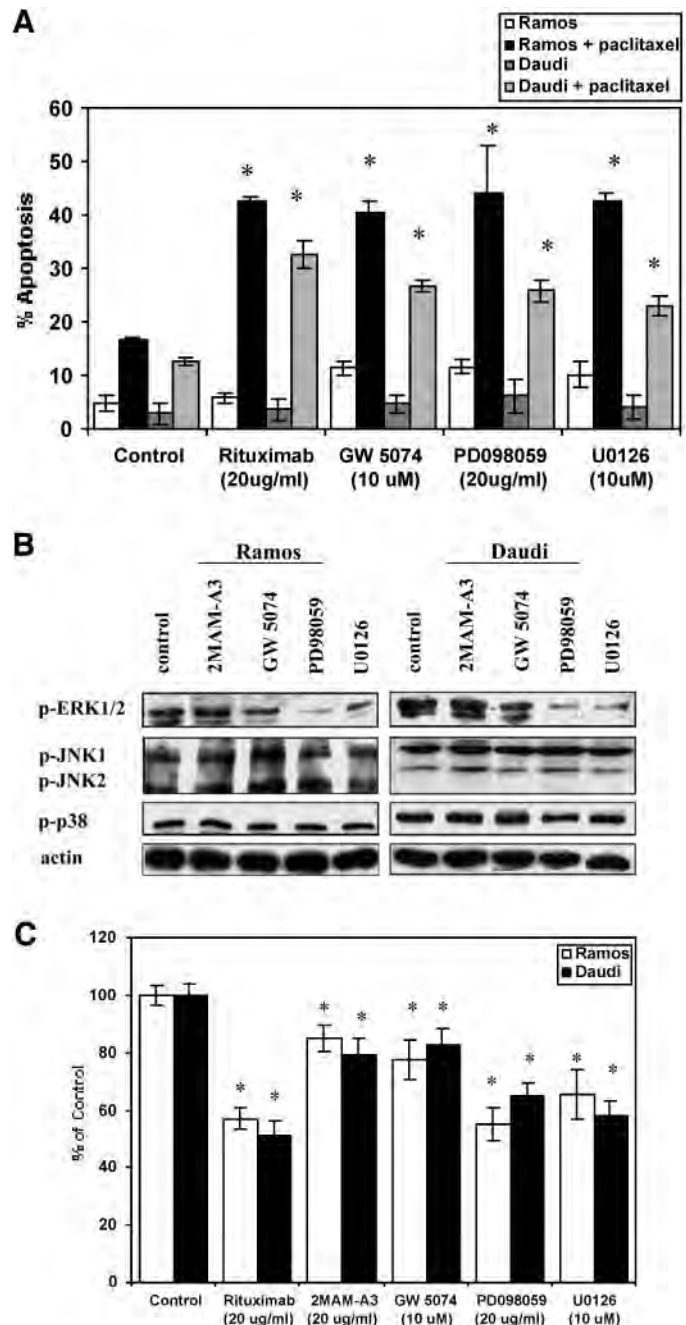

Fig. 3. Pharmacological inhibition of the ERK1/2 signaling pathway. *A*, sensitization of the Ramos and Daudi cells to paclitaxel-induced apoptosis. Tumor cells were either left untreated (control) or pretreated with GW5074 (10 μmol/L, 45 minutes), U0126 (10 μmol/L, 45 minutes), or PD098059 (20 μg/mL, 45 minutes). Thereafter, the cells were incubated with paclitaxel (10 nmol/L, 18 hours) and subjected to PI staining. *B*, specificity of the inhibitors for the ERK1/2 pathway. Tumor cells were treated with inhibitors under the conditions mentioned above [also treated with 2MAM-A3 (20 μg/mL, 7 hours]. Total cell lysates (40 μg) were used in immunoblotting experiments with Abs specific for pERK1/2, p-p38, and pJNK1/2 MAPKs. *C*, inhibition of cellular proliferation by rituximab and ERK1/2 inhibitors. An aliquot of the samples from *A* [10⁴ cells per sample; also treated with 2MAM-A3 (20 μg/mL, 7 hours)] was used in a 24-hour XTT proliferation assay to measure the proliferation rate of Ramos and Daudi cells on treatment with rituximab and the inhibitors. Results represent the mean ± SD of triplicate samples from two independent experiments. ∗, $P < 0.05$ (significant).

state of these signaling molecules was unaffected by the inhibitors (data not shown). Together, these results show that GW5074, PD098059, and U0126 specifically inhibit the ERK1/2 signaling pathway by decreasing the phosphorylation of ERK1/2. These results also show that pharmacological disruption of the ERK1/2 pathway using specific inhibitors, such as rituximab, sensitizes the cells to paclitaxel.

Inhibition of Cell Proliferation. Rituximab inhibits cellular proliferation of Ramos, Daudi, and other NHL B-cell lines (7, 8). We examined whether the ERK1/2 pathway affected by rituximab treatment was involved in the proliferation of the Ramos and Daudi cells. An aliquot of the samples from Fig. 3*A* (10⁴ cells per sample) was used in a 24-hour XTT assay to analyze the antiproliferative effects of rituximab and the inhibitors. The results presented in Fig. 3*C* demonstrate that specific pharmacological inhibitors of the ERK1/2 pathway mimic rituximab in decreasing the proliferation rate of the Ramos and Daudi cells.

ERK1/2 MAPK pathway; thus, alteration in the DNA binding activity of AP-1 on rituximab treatment was examined. The results demonstrate that AP-1 DNA binding activity was significantly reduced in the presence of rituximab as early as 3 to 6 hours after treatment, and that it remained decreased in the presence of rituximab during the entire experiment (24 hours). Rituximab-mediated decrease in AP-1 DNA binding activity was corroborated by the use of PD098059 (20 μg/mL, 45 minutes; Fig. 4*A*; refs. 29 and 30). Furthermore, the specificity of the EMSA assay was demonstrated by including crucial controls (*e.g.*, positive control, no nuclear extract, unlabeled free probe, and unrelated probe). Because the AP-1 complex is composed of Jun and Fos family members, supershift experiments using c-Jun and c-Foc Abs were performed. The postulated AP-1 bands showed significant shift on the addition of the Abs to the nuclear extracts, confirming the involvement of AP-1 (Fig. 4*A*).

Additional evidence for the direct role of rituximab and the ERK1/2 pathway in the regulation of Bcl-$_{xL}$ expression was provided by luciferase reporter assays. To this end, a 650-bp DNA fragment spanning the Bcl-x 5′ promoter region (Bcl-x WT) was inserted into pGL2-Basic luciferase plasmid (16). Using electroporation, the cells were transfected with this plasmid, and the cells were allowed to recover for 36 hours. The cells were then treated with either rituximab (20 μg/mL) or PD098059 (20 μg/mL) for an additional 18 hours. Then, the cells were harvested using 1× lysis buffer, and luciferase activity was measured with an analytical luminescence counter. Transfection with the WT promoter resulted in robust luciferase activity in both cell lines, albeit to a varying degree. However, treatment of the cells with rituximab or PD098059 substantially diminished the luciferase activity (Fig. 4*B*). These results corroborate the above-mentioned findings demonstrating the regulation of Bcl-$_{xL}$ by rituximab and the ERK1/2 pathway.

Based on the above-mentioned observations, we next examined whether rituximab and/or ERK1/2-specific inhibitors modulate Bcl-$_{xL}$ transcription. Tumor cells were either left untreated (control) or treated with rituximab (20 μg/mL, 1–24 hours), 2MAM-A3 (20 μg/mL), GW5074 (10 μmol/L), PD098059 (20 μg/mL), or U0126 (10 μmol/L), and total RNA was extracted and reverse transcribed to first-strand cDNA. Oligonucleotide primers specific for Bcl-$_{xL}$ mRNA were used in a PCR. As shown, 2MAM-A3 had no effect on Bcl-$_{xL}$ gene expression, whereas rituximab and the inhibitors decreased the transcription of Bcl-$_{xL}$ (Fig. 4*C*, *a*). Rituximab inhibited Bcl-$_{xL}$ transcription in a time-dependent manner, beginning as early as 1 hour after treatment, and the inhibition was more pronounced at later time points (Fig. 4*C*, *b*). Together, these results denote the ability of rituximab to inhibit the ERK1/2 pathway, decrease AP-1 DNA binding activity, and down-regulate Bcl-$_{xL}$ transcription.

Rituximab-Mediated Up-Regulation of RKIP Parallels Bcl-$_{xL}$ Down-Regulation

The above-mentioned data demonstrate that rituximab (and the inhibitors) inhibits the ERK1/2 pathway. Thus, we investigated the possible mechanism by which rituximab exerts this inhibitory effect. Recently, RKIP has been identified as a negative regulator of the ERK1/2 signaling pathway (14, 15). Therefore, we examined whether RKIP induction was associated with rituximab-mediated inhibition of the ERK1/2 pathway and chemosensitization of the Ramos and Daudi cells. We observed a time-dependent induction of RKIP in rituximab (20 μg/mL)-treated Ramos and Daudi cells. Intriguingly, the induction of RKIP coincided with Bcl-$_{xL}$ down-regulation (also observed as early as 3–6 hours; Figs. 5*A* and *B*).

It has been shown that RKIP interferes with the ERK1/2 pathway via physical interaction with Raf-1 (14, 15), thus we examined whether rituximab potentiates the association between Raf-1 and RKIP. Tumor cells were grown in the presence or absence of rituximab (20 μg/mL, 24 hours), and total cell lysates were used in an immunoprecipitation assay. Using anti–Raf-1 Ab, Raf-1 was precipitated, and the membranes were subsequently immunoblotted with anti-RKIP polyclonal Ab. As depicted (Fig. 5*C*), the association of RKIP with Raf-1 was significantly enhanced by rituximab. In addition, the lysates contained similar levels of Raf-1 and, consistent with findings in Fig. 5*A* and *B*, the rituximab-treated samples exhibited a higher level of RKIP (Fig. 5*C*). These results suggest that rituximab up-regulates RKIP and enhances the association of RKIP with Raf-1, events that can account for the inhibition of the ERK1/2 pathway.

DISCUSSION

We have reported previously that rituximab interferes with the intracellular signaling pathways and sensitizes various NHL cells to drug-induced apoptosis. The observed chemosensitization was due to the selective inhibition of antiapoptotic gene products Bcl-2 (in 2F7 ARL; ref. 7) and Bcl-$_{xL}$ (in non-ARL cells) by rituximab (8). The current study delineates the signaling pathway used by rituximab for selective inhibition of Bcl-$_{xL}$ in Ramos and Daudi cells. Rituximab decreases the phosphorylation-dependent state of the components of the ERK1/2 signaling pathway concomitant with the up-regulation of RKIP expression. Induction of RKIP enhances the physical association of RKIP with Raf-1, resulting in decreased activity of the ERK1/2 pathway, diminished AP-1 DNA binding activity, down-regulation of Bcl-$_{xL}$ expression, and subsequent chemosensitization of the cells. These events occurred with similar kinetics and were observed 3 to 6 hours after rituximab treatment. Using specific inhibitors corroborated rituximab-mediated inhibition of the ERK1/2 pathway and Bcl-$_{xL}$ expression. These findings reveal for the first time the interruption of the ERK1/2 pathway by rituximab.

The ERK1/2 pathway is constitutively activated in Ramos and Daudi cells (Fig. 2). Our findings demonstrate that monomeric rituximab is incapable of inducing apoptosis and inhibits the ERK1/2 pathway in Ramos and Daudi cells. However, previous reports have demonstrated activation of the ERK1/2, p38, and JNK1/2 MAPK pathways and induction of apoptosis by cross-linking rituximab (33, 34). In agreement with these reports, cross-linking rituximab (20 μg/mL + 50 μg/mL antihuman immunoglobulin) induced robust and sustained phosphorylation of ERK1/2 and p38 MAPK (Fig. 2*B*) and induced significant apoptosis in Ramos and Daudi cells (data not shown). Li *et al.* (35) identified novel mechanisms that may explain the opposing effects of p38 in apoptosis induction, whereby activation of p38 by mitogen-activated protein kinase kinase (MKK)3/6 was followed by rapid dephosphorylation of MEK1/2 and subsequent apoptosis. It is possible that cross-linking rituximab inhibits this pathway, whereas a different pathway is modulated by monomeric rituximab. These findings suggest that the pathways used by monomeric and cross-linked rituximab are different.

Because the constitutive activation of the ERK1/2 signaling pathway confers a chemoresistance phenotype on tumor cells and induces their rapid proliferation (36–38), inhibition of this pathway may confer drug sensitivity. Thus, interruption of this pathway is a target for therapeutic intervention for the treatment of leukemia and other tumors (36–38). Herein, we demonstrate that rituximab inhibits the ERK1/2 pathway and sensitizes the cells to drug-induced apoptosis. The phosphorylation-dependent state of Raf-1, MEK1/2, and ERK1/2 is significantly decreased 3 to 6 hours after rituximab treatment in NHL B cells, concomitant with inhibition of MEK1/2 kinase activity (Fig. 2). Moreover, rituximab sensitized these cells to paclitaxel (Fig. 1*A*), suggesting a novel function for rituximab as a negative regulator
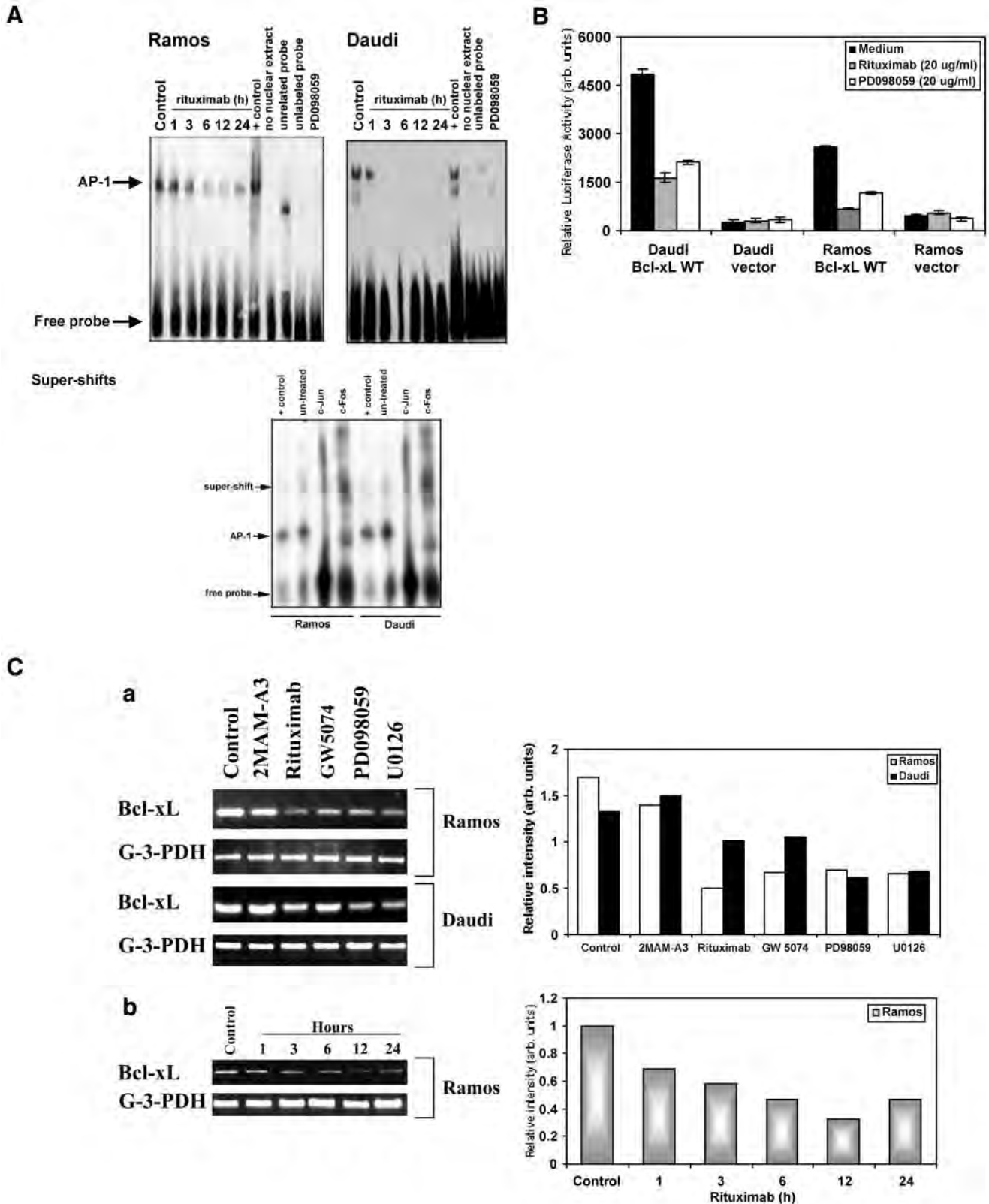
Fig. 4. Rituximab diminishes constitutive AP-1 DNA binding activity and Bcl-$_{xL}$ gene expression. *A,* After overnight growth in RPMI 1640 + 1% fetal bovine serum, Ramos and Daudi cells were washed and grown in either complete medium (control) or complete medium supplemented with rituximab (20 μg/mL; 1, 3, 6, 12, and 24 hours) or PD098059 (20 μg/mL, 45 minutes). Ten micrograms of nuclear lysates were subjected to EMSA. Specificity of the assay was confirmed by the inclusion of appropriate controls. For the supershifts, 1 μL of the appropriate Abs was added to the nuclear extracts 20 minutes before the addition of the labeled probe. *B,* A Bcl-x promoter fragment spanning the −640 to +9 region relative to the transcriptional start site was cloned into pGL2-Basic luciferase reporter vector (16). The cells were then transfected with 10 μg of this reporter plasmid or empty vector. Thirty-six hours after transfection, the cells were either left untreated or treated with PD098059 (20 μg/mL) or rituximab (20 μg/mL). The samples were harvested after 18 hours and assessed for luciferase activity. *C,* Tumor cells were either left untreated or treated with the inhibitors [rituximab (20 μg/mL), GW5074 (10 μmol/L), PD98059 (20 μg/mL), or U0126 (10 μmol/L)], and total RNA was extracted and reverse transcribed to first-strand cDNA. Complementary DNA (2.5 μg) of various sample conditions was used in PCR analysis using Bcl-$_{xL}$–specific primers. The intensity of the bands was normalized to the levels of the corresponding glyceraldehyde-3-phosphate dehydrogenase (*G-3-PDH*). The results are representative of two independent experiments.
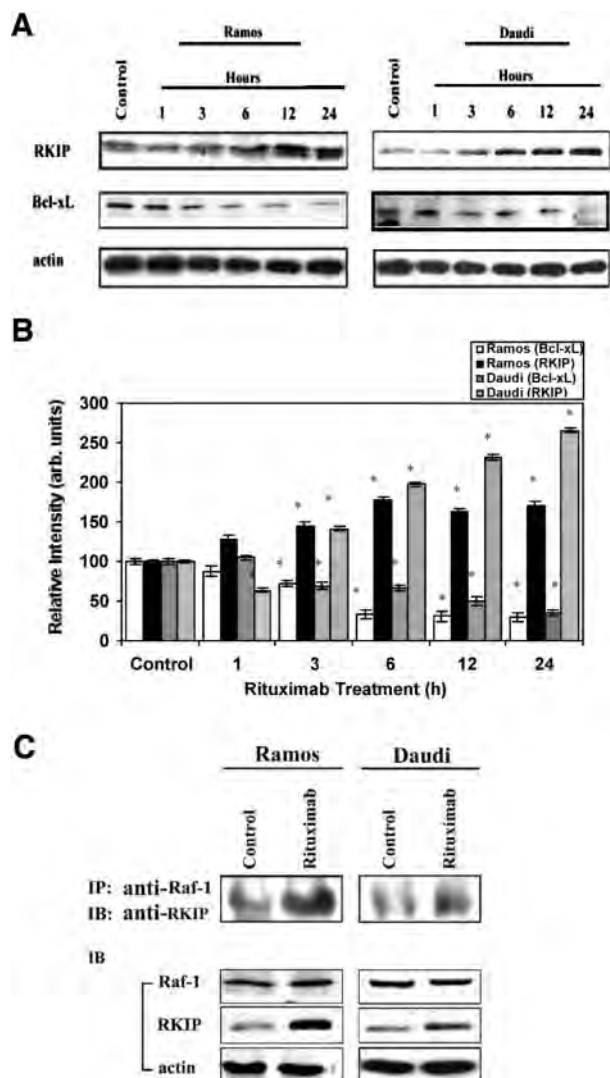
Fig. 5. Rituximab induces RKIP expression and augments physical association of RKIP with Raf-1. *A*, Ramos and Daudi cells were grown in either complete medium or complete medium supplemented with rituximab (20 μg/mL) for various time periods (1–24 hours). At the end of the incubation periods, cells were lysed and subjected to immunoblot analysis. *B*, Densitometric analysis shows significant alterations in RKIP and Bcl-$_{xL}$ expression on rituximab exposure. *C*, immunoprecipitation of Raf-1 with RKIP on treatment with rituximab (20 μg/mL, 24 hours). Total cell lysates were used in an immunoprecipitation assay. Raf-1 was precipitated, and the membranes were immunoblotted with anti-RKIP polyclonal Ab. The results are representative of three independent experiments. *, $P < 0.05$ (significant).

of the ERK1/2 pathway. This was corroborated by pharmacological interruption of the ERK1/2 pathway using specific inhibitors, which also sensitized the cells to paclitaxel at levels comparable with those achieved by rituximab (Fig. 3*A*). Our results corroborate previous reports in which MEK inhibition synergized with UCN-01 (39) and augmented the apoptotic effects of paclitaxel (40, 41). In our studies, pretreatment with rituximab followed by paclitaxel was optimal for sensitization, and pretreatment with the ERK1/2 inhibitors followed by paclitaxel mimics the effects of rituximab. Thus, inhibition of the ERK1/2 pathway seems to be required before paclitaxel treatment to trigger apoptosis in this model. However, studies by Yu *et al.* (41) in the U937 leukemia cell line demonstrated that pretreatment with the inhibitors followed by paclitaxel did not sensitize the cells to paclitaxel. The discrepancy may be due to differences in the cell lines used as well as differences in the duration of paclitaxel treatment (6 hours in their studies *versus* 18 hours in the present study) or the activation status of the proteins in the signaling cascade.

Rituximab inhibits the proliferation rate of NHL B-cell lines through an unknown mechanism (7, 8). The ERK1/2 pathway is implicated in the proliferation of tumor cells (36, 38). Here we demonstrate that rituximab inhibits the constitutive activation of the ERK1/2 pathway in Ramos and Daudi cells and also reduces the rate of proliferation (Fig. 3*C*). Therefore, these findings suggest a link between rituximab-mediated inhibition of proliferation and inhibition of the ERK1/2 pathway. However, inhibition of proliferation cannot be explained solely by the inhibition of Bcl-$_{xL}$ because 2MAM-A3 did not reduce the proliferation rate to the levels achieved by rituximab.

We have established several lines of evidence for the involvement of the ERK1/2 pathway in the regulation of Bcl-$_{xL}$ expression. First, robust luciferase reporter activity was observed on transfection of the cells with WT Bcl-$_{xL}$ promoter, which was significantly reduced by rituximab and PD098059 (Fig. 4*B*). Secondly, specific inhibitors of the ERK1/2 pathway inhibited Bcl-$_{xL}$ transcription (Fig. 4*C*) and sensitized the cells to drug-induced apoptosis at levels comparable with those achieved by rituximab (Fig. 3*A*). Expression of Bcl-$_{xL}$ is regulated by several transcription factors including Ets, nuclear factor κB, signal transducers and activators of transcription, and AP-1 (31). Conversely, on activation, the ERK1/2 pathway leads to the activation of various transcription factors including AP-1. Mutational analysis has implicated AP-1 in the regulation of Bcl-$_{xL}$ (42); however, our data do not establish a direct role of AP-1 in the regulation of Bcl-$_{xL}$ expression. Our findings establish a correlation between rituximab-mediated inhibition of AP-1 DNA binding activity and inhibition of the ERK1/2 pathway and Bcl-$_{xL}$ down-regulation. Nonetheless, the likelihood of the contribution of other transcription factors to the regulation of Bcl-$_{xL}$ by rituximab has not been ruled out.

The protective role of Bcl-$_{xL}$ against chemotherapy-triggered apoptosis (11, 12) was confirmed by using 2MAM-A3, which binds to Bcl-$_{xL}$ at the hydrophobic groove formed by the highly conserved BH1, BH2, and BH3 domains, thus impairing the antiapoptotic ability of Bcl-$_{xL}$ (19). Although unable to regulate transcription or translation of Bcl-$_{xL}$, 2MAM-A3 sensitized the cells to paclitaxel (and other drugs; data not shown), at levels comparable with those achieved by rituximab, via functional impairment of Bcl-$_{xL}$. These findings support our contention that down-regulation of Bcl-$_{xL}$ expression by rituximab is critical for chemosensitization.

Bcl-$_{xL}$ is abundantly expressed in lymphomas (10) and protects the cells from apoptosis induced by DNA-damaging agents and metabolic, microtubule, and topoisomerase inhibitors (11, 12). An inverse correlation between Bcl-$_{xL}$ levels and sensitivity to 122 standard anticancer agents has been established (12). Also, Bcl-$_{xL}$ acts independently of wild-type p53 function or cell type and, through modulation of apoptosis, plays a major role in the determination of cellular response to a wide variety of drugs (9–13). Our results suggest that in Ramos and Daudi cells, Bcl-$_{xL}$ is the main antiapoptotic factor, and the ability of rituximab to negatively modulate the expression of Bcl-$_{xL}$ may explain its effectiveness in combination with chemotherapy in the treatment of some cases of NHL. This contention was further supported by using Bcl-$_{xL}$–overexpressing cells, which expressed higher resistance against a battery of structurally and functionally diverse antineoplastic agents (cisplatin, Adriamycin, etoposide, and paclitaxel; data not shown). Functional impairment of Bcl-$_{xL}$ (by 2MAM-A3) sensitized these cells to paclitaxel. Rituximab was only able to reduce the endogenous levels of Bcl-$_{xL}$ in these cells and was not able to reduce the ectopically expressed Bcl-$_{xL}$ driven by the overexpressing plasmid, and it exerted a modest sensitizing attribute, suggesting that the level of Bcl-$_{xL}$ is critical for drug-resistance. Our findings with Bcl-2–expressing Daudi and Bcl-2–deficient Ramos cells reveal that rituximab-mediated chemosensitization is independent of Bcl-2 expression, which is in agreement with recent findings

Fig. 6. Proposed model of rituximab-mediated inhibition of the ERK1/2 MAPK pathway and sensitization of NHL B cells to paclitaxel-induced apoptosis. The ERK1/2 signaling pathway is constitutively active in Ramos and Daudi cells, and these cells express low levels of RKIP. On ligation to CD20, rituximab up-regulates RKIP expression. RKIP blocks the phosphorylation and activation of Raf-1 via physical interaction and renders it incapable of relaying the signal to the downstream components of the signaling cascade. This will in turn decrease MEK1/2 and ERK1/2 phosphorylation and MEK1/2 kinase activity. Subsequently, the DNA binding activity of transcription factor AP-1 that is under the regulation of the ERK1/2 pathway is diminished. Deactivation of the ERK1/2 MAPK pathway will decrease the proliferation rate of the tumor cells; diminish the levels of Bcl-$_{xL}$, which will decrease the apoptosis threshold; and sensitize the NHL B cells to paclitaxel. Pharmacological inhibition of the ERK1/2 MAPK pathway (by GW5074, PD098059, and U0126) or functional impairment of Bcl-$_{xL}$ (by 2MAM-A3) mimics the antiproliferative and chemosensitizing attributes of rituximab.
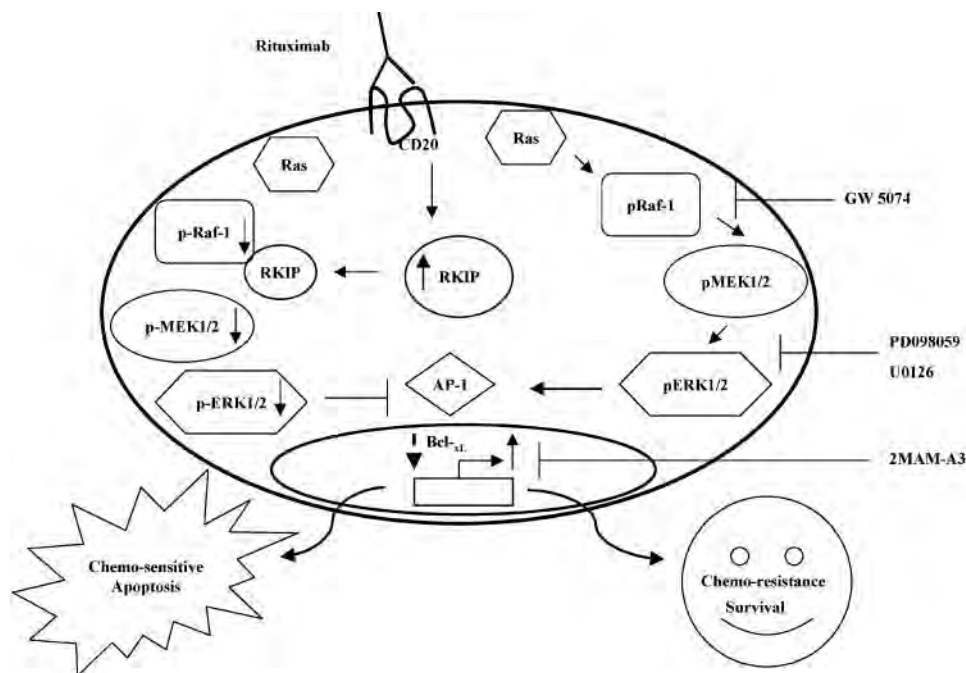
(30, 43). Bcl-$_{xL}$ down-regulation by rituximab is most likely cell type specific because it was not observed in the 2F7 ARL cells (7).

The inhibition of the ERK1/2 pathway might occur via several different mechanisms. It might be through the de-activation of the src family kinase Lyn. Indeed, a decrease in p-Lyn by rituximab was observed (data not shown). Alternatively, it might be due to the modulation of RKIP expression that inhibits the ERK1/2 pathway (14, 15). In fact, our findings reveal that rituximab up-regulates the expression of RKIP and facilitates the association of RKIP with Raf-1 (Fig. 5). Physical association between RKIP and Raf-1 will abrogate the ability of Raf-1 to phosphorylate and activate downstream molecules such as MEK1/2 and ERK1/2. *In vitro*, RKIP disrupts the interaction between Raf-1 and MEK, thus behaving as a competitive inhibitor for MEK, and inhibits AP-1–dependent gene expression by suppressing the ERK1/2 pathway (14, 15). Our results corroborate these findings and demonstrate that rituximab-mediated RKIP induction diminishes the phosphorylation of the components of the ERK1/2 pathway, reduces AP-1 DNA binding activity, and decreases Bcl-$_{xL}$ transcription and translation, all of which occur with similar kinetics. These findings provide a novel mechanism induced by rituximab that regulates cell survival and sensitizes the cells to paclitaxel through induction of RKIP and inhibition of the ERK1/2 pathway. Thus, RKIP expression regulates drug sensitivity. The novel and important role of RKIP as a mediator of drug-induced apoptosis is not limited to the NHL model. Ectopic expression of RKIP sensitizes drug-resistant cells to undergo apoptosis (44). Furthermore, down-regulation of RKIP expression in tumor cells confers drug resistance by releasing its inhibitory constraint of ERK1/2 and nuclear factor κB major survival pathways (14, 15, 45). The mechanism by which rituximab and other chemotherapeutic agents regulate RKIP expression, however, is unknown and under investigation. The role of RKIP in the regulation of cell survival and apoptosis in cancer cells may be clinically important. For instance, a novel antimetastatic function for RKIP in prostate cancer has recently been proposed (46), showing the involvement of the ERK1/2 pathway in tumor progression and metastasis and further confirming the specific interaction of RKIP with the ERK1/2 pathway. Notably, the invasion of tumor cells was abrogated only in the presence of PD098059. Thus, the modulation of

RKIP expression in cancer cells may dictate the outcome of tumor progression and response to apoptosis-inducing stimuli.

In conclusion, we have described a novel mechanism by which rituximab affects both proliferative and apoptotic signaling pathways schematically represented in Fig. 6. Accordingly, rituximab up-regulates RKIP expression and interferes with the constitutively active ERK1/2 pathway, resulting in diminished AP-1 DNA binding activity and Bcl-$_{xL}$ expression. Decreased levels of Bcl-$_{xL}$ will in turn lower the apoptosis threshold, and the cells will be sensitized to drug-induced apoptosis through the type II mitochondrial pathway (8). Pharmacological interruption of the ERK1/2 pathway (*e.g.,* GW5074, PD098059, and U0126) or functional impairment of Bcl-$_{xL}$ (*e.g.,* 2MAM-A3) mimics the antiproliferative and chemosensitizing effects of rituximab. Hence, this study identifies several potential targets for therapeutic intervention (namely, the components of the ERK1/2 pathway, Bcl-$_{xL}$, and RKIP) and might provide a rational molecular basis for the therapeutic use of rituximab and/or ERK1/2 inhibitors in combination with chemotherapeutic compounds.

ACKNOWLEDGMENTS

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Expression of transcription factor Yin Yang 1 in prostate cancer

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Abstract. The transcription repressor Yin Yang 1 (YY1) is expressed in several human cancer cell lines and its expression correlates with resistance to immune-mediated apoptosis. This study used tissue microarrays to investigate the expression and localization of YY1 in 1364 representative tissue samples from 246 hormone naïve prostate cancer patients who underwent radical prostatectomy. Staining intensity and frequency measures for both YY1 nuclear and cytoplasmic expression were higher in neoplastic tissues and in PIN samples compared to matched benign cells (p<0.0001 for all comparisons). Expression of YY1 is predominantly elevated in early malignancy (PIN), as well as in tumors of intermediate to high morphologic grade (Gleason's grade 3-5). Using multivariate Cox proportional hazards analysis, we observed that low nuclear YY1 staining is an independent predictor of a shorter time to recurrence (p=0.012). Based on these results, we hypothesize that YY1 may play a role in prostate cancer development; however, decreased YY1 may give metastatic cells a survival advantage. These results may also implicate YY1 as a useful diagnostic and prognostic marker.

Introduction

Prostate cancer is the most common cancer of males in the U.S., with an age-adjusted incidence of 170 per 100,000 and 29,900 associated deaths estimated for 2004 (1,2). Sixteen percent of patients with localized invasive prostate cancer who undergo radical prostatectomy surgery will develop recurrence of malignancy within 5 years (3). When detected at locally advanced or metastatic stages, no consistently curative treatment regimen exists. Treatment for metastatic prostate cancer includes hormonal ablation, chemotherapy and combination therapies. Unfortunately, there is frequent relapse of an aggressive androgen-independent disease that is insensitive to further hormonal manipulation or to treatment with conventional chemotherapy (4). Therefore, there is a need for alternative therapies.

One such alternate approach is immunotherapy. This strategy depends on enhancing the recognition of tumor cells by components of the immune system including cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (5-11). This strategy also predicts that tumors which have become resistant to chemotherapeutic drugs, may still be targets for NK or CTL-mediating killing. However, this approach has only been modestly successful in part due to acquired cross-resistance by tumor cells to immune-mediated surveillance thus ultimately leading to tumor progression and metastasis of the resistant cells (12). The mechanism(s) responsible for the anti-apoptotic phenotype, if identified, may be both a useful prognostic indicator as well as an important therapeutic target.

Our recent findings reveal a novel mechanism of tumor cell resistance to immune-mediated cytotoxicity. We show that resistance to Fas-mediated apoptosis of ovarian and prostate cancer cells is in large part due to the transcription repressor Ying Yang 1 (YY1) that inhibits Fas expression. The inhibition of YY1 up-regulates Fas expression and the cells become sensitive to Fas-mediated apoptosis (13).

YY1 is a multifunctional DNA binding protein, which can activate, repress or initiate transcription depending on the context in which it binds (14,15). In addition, YY1 can

modulate protein levels or activity through protein-protein interaction (16). Through DNA binding and/or protein interaction YY1 has been identified as a potential repressor factor for several genes which include human interferon-γ (17,18), IL-3 (19), Fas (13), GM-CSF (17,20) and p53 (16). Significantly, we have identified a relevant putative repressor cluster at the silencer region of the human Fas promoter that matched the consensus sequence that binds the transcription factor YY1 (13).

To start to address the role of YY1 in regulating the sensitivity of prostate cancer cells to apoptosis, we have initiated a study to characterize the expression level and location of this factor in normal and malignant prostate cancer cells. Preliminary findings have demonstrated a relatively high level of YY1 in the human prostate cell line PC3, and in a limited studies, increased expression of YY1 in malignant compared to non-malignant human prostate tissue. Here we have constructed and utilized a high density prostate tissue array to more fully characterize the level and subcellular localization (i.e., cytoplasmic versus nuclear) of YY1 during different stages of malignant progression. Notably, our results strongly indicate that YY1 expression potentially has both diagnostic and prognostic values for prostate cancer.

Materials and methods

Western blot analysis. PC-3 cells were obtained from the ATCC (Manassas, VA) and cultured as a monolayer in RPMI-1640 (Life Technologies, Bethesda, MD) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies). Cells were lysed at 4˚C in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl], and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN). Protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2X SDS sample buffer [6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mecrapto-ethanol, 10% glycerol and 0.02% bromophenol blue] and boiled for 10 min. The cell lysates (40 μg) were then electro-phoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (21). The mouse anti-YY1 antibody was purchased from Geneka Biotechnology (Montreal, Quebec, Canada), and the mouse monoclonal anti-ß-actin was purchased from Chemicon (Temecula, CA). Levels of ß-actin were used to normalize the YY1 expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images using public domain NIH Image J Program (http://rsb.info.nih/ij/).

Prostate tissue microarray. The prostate tissue microarray (TMA) was constructed using formalin-fixed, paraffin-embedded prostate tissue samples provided through the Department of Pathology and Laboratory Medicine at the UCLA Medical Center under IRB approval. Primary radical prostatectomy cases from 1984-1995 were randomly selected from the pathology database. The original H&E stained slides were reviewed by a pathologists (D.S.) utilizing the Gleason histological grading (22) and the 1997 AJCC/UICC TNM classification systems (23). Case material from 246 prostate-ctomies was arrayed into 3 blocks encompassing a total of 1,364 individual tissue cores. All cases were of the histo-logical type adenocarcinoma, conventional, not otherwise specified (24).

TMAs were constructed as previously described (25). At least 3 replicate tumor samples were taken from donor tissue blocks in a highly representative fashion. Tumor samples were accompanied by matching benign (morphologically normal or hypertrophic) and *in situ* neoplastic lesions (PIN), when available. Table I shows the clinicopathologic data for the 190 patients included in the outcomes analysis. The median age at the time of surgery was 65 (range 46-76). One hundred and twelve patients (59%) were low grade (Gleason score 2-6); 78 (41%) were high grade (Gleason score 7-10). Approximately half of the tumors (51%) were confined to the prostate (organ confined here = T2a or T2b with negative lymph nodes, no capsular extension and with negative surgical margins). One hundred and twenty-eight (67%) patients were margin negative, 62 (33%) margin positive and 32 (17%) had seminal vesicle invasion (pT3b). Regarding capsular invasion, 44 (23%) had no invasion, 107 (56%) had invasion and 39 (21%) had capsular extension. Concurrent regional lymphadenectomy accompanied 187 (98%) cases, only 9 of which (5%) were positive for metastases. The maximum pre-operative serum PSA was known for 169 patients (89%), with a median value of 8.9 ng/ml (range 0.6-76.0).

A retrospective analysis for outcome assessment was based on detailed anonymized clinicopathologic information linked to the TMA tissue specimens. Recurrence, defined as a postoperative serum PSA of 0.2 ng/ml or greater, was seen in 65 (34%) patients. Total follow-up, defined as the time to recurrence or to last contact in non-recurring patients, had a median of 49.5 months (range 0.1-163). The median follow-up time within the recurring and non-recurring groups was 21 (1.0-115) and 66 months (range 0.1-163), respectively.

Each case was represented by an average of 3.2 informative tumor spots. Tissue spots from all 246 cases were included in the histological distribution analysis of YY1; 79% of these spots were informative (i.e., contained benign and/or malignant epithelial cells). Patients that were treated preoperatively with neoadjuvant hormones were excluded from the clinical analysis (n=20). An additional 23 cases were not evaluated predominantly due to a lack of target tumor tissue. For thirteen cases, we had no associated outcome data. Therefore, of 246 total cases, 190 (77%) were available for outcome studies.

Immunohistochemistry. A standard 2-step indirect avidin-biotin complex (ABC) method was used (Vector Laboratories, Burlingame, CA). Tissue array sections (4-μm thick) were cut immediately prior to staining using the TMA sectioning aid (Instrumedics, NJ). Following deparaffinization in xylenes, the sections were rehydrated in graded alcohols and endo-genous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed in 95ºC solution of 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval, and then blocked with 5% normal goat serum for 30 min. Endogenous biotin was blocked with sequential application of avidin D then biotin (A/B blocking system, Vector Laboratories). Primary rabbit anti-human YY1 poly-

Table I. Relationship of YY1 nuclear expression with clinicopathologic parameters.

YY1 nuclear expression frequency	All patients		'Low YY1' (% of total)		'High YY1' (% of total)		P-value[a]
Total cases (190)	42	(22)	148	(78)			
Age at surgery							
Median	65 (range 46-76)		65 (range 50-76)		65 (range 46-74)		0.12[b,c]
Gleason score							0.65[c]
2-6	112	(59)	23	(55)	89	(60)	
7-10	78	(41)	19	(45)	59	(40)	
Pathology pT stage[d]							0.51[c]
PT2-pT3a	158	(83)	33	(79)	125	(84)	
PT3b	32	(17)	9	(21)	23	(16)	
Lymph node status							0.42[b,c]
Positive	9	(5)	1	(2)	8	(5)	
Negative	178	(95)	40	(98)	138	(95)	
Tumor margins							0.94[c]
Positive	62	(33)	13	(31)	49	(33)	
Negative	128	(67)	29	(69)	99	(67)	
Capsular invasion							0.23[c]
No invasion	44	(23)	6	(14)	38	(26)	
Invasion	107	(56)	28	(67)	79	(53)	
Extension	39	(21)	8	(19)	31	(21)	
Organ confined[e]							0.74[c]
Yes	97	(51)	20	(48)	77	(48)	
No	93	(49)	22	(52)	71	(52)	
High risk (n=187)							0.79[c]
Yes	36	(19)	9	(22)	27	(18)	
No	151	(81)	32	(78)	119	(82)	
PSA ng/ml[f] (n=169)							
Median (range)	8.9 (0.6-76.0)		9.1 (1.8-76.0)		8.9 (0.6-60.7)		
Mean	13.3		15.8		12.6		0.47[b,c]
Recurrence[g]							0.024[h]
Yes	65	(34)	21	(50)	44	(30)	
No	125	(66)	21	(50)	104	(70)	
Follow-up[i]							
Median (range)	49.5 (0.1-163.0)		43.0 (1.0-120.0)		51.0 (0.1-163.0)		
Mean	53.5		46.7		55.4		0.21[b,c]

[a]P-value was determined by the Pearson χ^2-test with Yates continuity correction unless otherwise specified. [b]P-value was determined by the Mann-Whitney test. [c]Not signficant. [d]pT3b indicates seminal vesicle invasion. There are no pT4 cases. [e]Organ confined, no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative. [f]Preoperative PSA values. [g]Recurrence, PSA elevation raising >0.2 ng/ml status post radical prostatectomy. [h]As a continuous variable, YY1 minimum positivity is associated with recurrence by logistic regression; (p=0.0097; 0.99; 95% confidence interval 0.98-0.99). [i]Follow-up, total time (months) to recurrence or last follow-up.

clonal IgG_1 antibody (Geneka Biotechnology, Inc.) was applied at a 1:1,000 dilution (0.2 µg/ml) for 60 min at room temperature. After washing, biotinylated goat anti-rabbit IgG (Vector Laboratories) was applied for 30 min at room temperature. The ABC complex was applied for 25 min followed by the chromogen diaminobenzidine (DAB). PBS (10 mM, pH 7.4) was used for all wash steps and dilutions. Incubations were performed in a humidity chamber. The sections were counterstained with Harris' hematoxylin, followed by dehydration and mounting.

Antibody specificity was tested by concentration-dependent inhibition of staining using the immunizing YY1 peptide (Geneka Biotechnology, Inc.). Anti-YY1 antibody was preincubated for 3 h at room temperature with a 0X, 5X or 10X molar excess of peptide. The antibody in the presence or absence of the peptide was then added to a mini-prostate array (16 spots) and stained as described above.

Scoring of immunohistochemistry. Semi-quantitative assessment of antibody staining on the TMAs was performed

by a study pathologist (A.R.) blinded to the clinicopathologic variables. Random spots were double scored for quality control purposes by one of the study pathologists (D.S.). The target tissue for scoring was the prostatic glandular epithelium, scoring of benign tissues did not include basal cells. Tissue spot histology and grading was confirmed on H&E stained TMA slides, as well as on the counterstained study slides. The staining intensities of the nuclear and cytoplasmic cellular compartments were scored separately, each on a 0-3 scale (0, negative; 1, weak; 2, moderate; 3, strong staining) as previously described (26). In addition, the frequency of positive target cells (range 0-100%) at each intensity level was also scored for each TMA spot.

For outcome analyses, we considered all 190 tumor cases for which we had recurrence data. For this analysis, the percentage of tumor cells staining (i.e., frequency) was first quantified for each tissue spot. Next, within these cases, tissue spots that had the lowest percentage of YY1 expression, were most statistically predictive of outcome.

Furthermore, the staining data were examined both as a continuous variable and as dichotomized variables using cut-off values. In this study, we primarily used the continuous, undichotomized staining scores ranging from 0-100% to draw statistical inferences. We also report our findings using a dichotomized staining score which optimally stratified the patient population into early and later recurring groups. The log-rank p-values that result from using the dichotomized variables should be interpreted as descriptive measures. Specifically, for the dichotomized data, we divided cases based on spots in which ≤50% of the cells expressed nuclear YY1 (referred to as 'YY1-low' cases; n=42) or in which >50% of cells expressed nuclear YY1 (referred to as 'YY1-high' cases; n=148).

To allow for comparisons across institutions, standardized tumor marker staining and scoring protocols should be established. We find that within our institution, the staining score is highly reproducible; however, the reproducibility of the staining score procedure across institutions needs to be confirmed.

Statistical analysis. Associations between YY1 expression groups and clinicopathologic variables were tested using the Pearson χ^2 test when dealing with a categorical variables and the Mann-Whitney test when dealing with an ordinal variable. The Mann-Whitney and the Kruskal-Wallis test are non-parametric two group and multi-group comparison tests, respectively. We used the Pearson correlation and corresponding p-values to relate two quantitative (interval scale) covariates (e.g., when studying the relationship between nuclear and cytoplasmic staining intensities and frequencies). Recurrence was defined as a rising total PSA >0.2 ng/ml status post prostatectomy, and time to recurrence was calculated from the date of the primary surgery. Patients without recurrence at last follow-up were censored. Kaplan-Meier plots were used to visualize recurrence-free time distributions and the log-rank test was used to test for differences between them. To assess which covariates associate with recurrence-free time, we fit both univariate and multivariate Cox proportional hazards models. The proportional hazards assumption was checked through the use of Schoenfeld residuals. For each
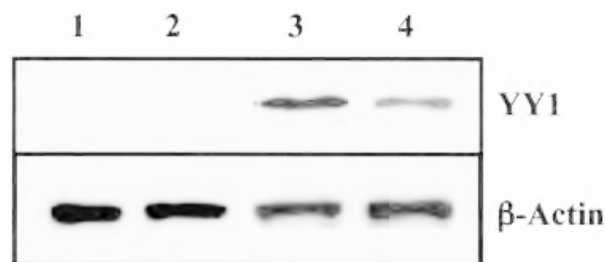

Figure 1. Western blot, PC-3 cell line. PC-3 cells were grown in RPMI with 10% of FBS. Total cellular protein was extracted from the culture and then separated by SDS-PAGE and transferred onto the nitrocellulose membrane as described in Materials and methods. The membrane was stained with medium alone (lane 1), IgG non-immune control (1:1,500; lane 2), anti-YY1 antibody (1:1,500 dilution, lane 3) and anti-YY1 antibody (1:3,000; lane 4). The ß-actin antibody (1:10,000) was used as a loading control. The findings revealed that PC-3 express YY1 constitutively. This experiment was repeated 3 times with similar results.

covariate, we list the 2-sided p-value, the hazard ratio and its 95% confidence interval. A p<0.05 was accepted as significant. All statistical analyses were carried out using either freely available software package R (http://www.r-project.org) or StatView Version 5.0 (SAS Institute Inc., Cary, NC).

Results

YY1 expression in PC3. YY1 is a transcription factor that demonstrates context-specific repression or activation activity (14). Recently, we have demonstrated that nitric oxide indirectly up-regulates the expression of Fas by blocking the silencing effect of YY1 (13). The apparent role of YY1 in modulating Fas expression, combined with postulated role of TNF receptor family members in tumor progression and resistance (11,27), prompted us to examine the expression distribution of YY1 in normal and malignant prostate tissue. To initiate this study, we first examined the expression of YY1 in an androgen-independent human prostate cancer cell line, PC3, by Western blot then immunocytochemical analyses. For Western blot analysis, cell extracts were prepared, electrophoresed, transferred and probed as described in Materials and methods. Abundant YY1 expression was detected in these cells as demonstrated by a prominent 68 kDa band (Fig. 1). Immunocytochemical results were consistent with Western blot data as ≥95% of the cells expressed YY1, predominantly within the nucleus (Fig. 2). These findings also established a positive control for subsequent immunohistochemical analyses in whole prostate tissues and tissue microarrays.

YY1 expression in prostate tissue sections. The relatively high expression in PC3 cells prompted us to embark on a study examining YY1 expression in human prostate tissue. We first examined YY1 expression by immunohistochemistry in three morphologically benign (normal and BPH) human prostate whole tissue sections. Staining was observed in the glandular epithelium, basal cells, and occasionally in stromal fibromuscular cells; Fig. 3A shows a representative example. Approximately 90% of the prostatic epithelium stained positive with typically weak to moderate intensities. Staining
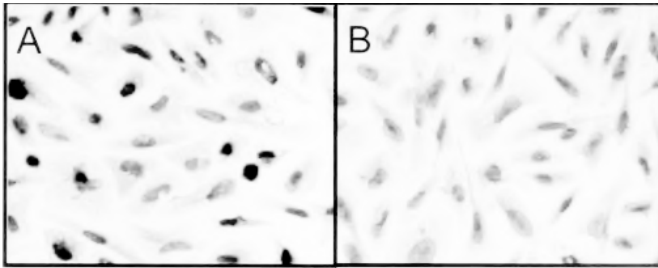
Figure 2. YY1 protein expression in a prostate cancer cell line (PC3). Distinct nuclear and light cytoplasmic staining of YY1 protein is seen by immuno-histochemistry (A). Replacing primary anti-YY1 antibody with non-immune pooled rabbit IgG at an equivalent concentration serves as negative control (B), note a complete absence of staining. x400 magnification.
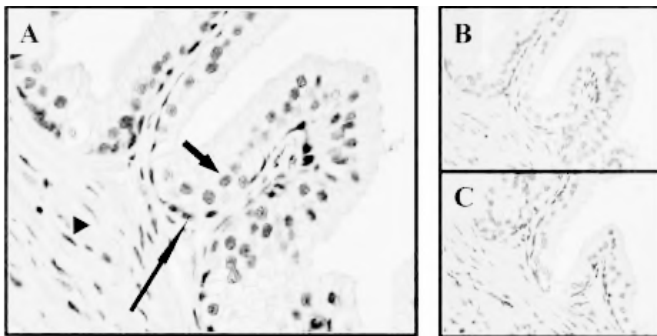

Figure 3. Typical YY1 protein expression localization, normal prostate whole tissues. Demonstration of the typical staining pattern of YY1 protein by immunohistochemistry (A), showing predominantly nuclear staining of glandular (thick arrow) and basal cells (thin arrow), as well as stromal fibromuscular cells (triangle). Negative controls include non-immune IgG primary antibody substituted for YY1 (B), and primary YY1 antibody staining after competitive inhibition with immunogen peptide (C). x400 magnification.

Figure 4. Spectrum of YY1 protein expression patterns in prostate cancer. Immuno-histochemical staining for YY1 protein is seen on prostate tissue samples. (A), Normal tissue included for comparison shows crisp diffuse nuclear staining; (C), High-grade tumor with finely granular nuclear staining; (E), Low-grade tumor with nuclear and diffuse cytoplasmic staining, note the normal gland in the lower left (arrow) showing nuclear staining only; (G), High-grade tumor with neuroendocrine features showing coarsely granular nuclear and diffuse cytoplasmic staining; (I), Low-grade tumor with minimal to absent nuclear staining. (B, D, F, H, J are all non-immune pooled rabbit IgG negative controls). x400 magnification.

was predominantly in the nucleus consistent with the expression pattern seen in PC3 cells (Fig. 3A). Negative control samples were incubated with non-immune sera had no staining (Fig. 3B). In addition, preincubation of the anti-YY1 antibody with varying doses of immunogen peptide displayed a dose-dependent inhibition of staining culminating in complete inhibition (Fig. 3C).

We next examined the spectrum of YY1 expression patterns on whole tissue sections from a panel of ten human prostate carcinomas (Fig. 4). Compared to the typically pronounced nuclear staining seen in non-malignant epithelium (Fig. 4A), two low-grade tumors demonstrated weak or minimal YY1 staining (example in Fig. 4I) while another low-grade tumor exhibited strong nuclear staining and diffuse cytoplasmic staining (Fig. 4E). Two high-grade tumors were also examined; one demonstrated weak to moderate nuclear staining (Fig. 4C), while the other showed relatively strong nuclear and cyto-plasmic staining (Fig. 4G). This complex set of staining patterns prompted us to examine a larger sample population using tissue microarray (TMA) technology.

YY1 expression is increased in malignant prostate samples. We next evaluated the protein expression of YY1 in clinical prostate samples using a TMA platform. We examined YY1
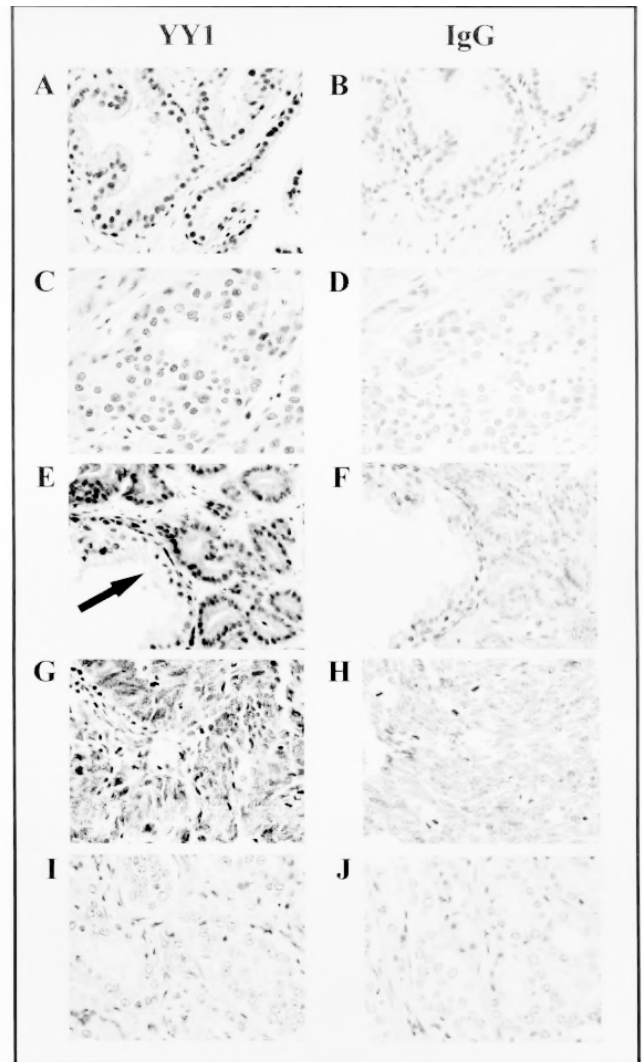
expression across histological categories on 1,061 informative primary site tissue spots (data for 12 lymph node metastases were not included).

Nuclear YY1 staining. Fig. 5A and B show distribution graphs of nuclear YY1 staining intensity (i.e., percentage of array spots with negative-weak or moderate-strong nuclear YY1 staining) and staining frequency (i.e., percentage of array spots that showed 0-49 or 50-100% of the cells positive for nuclear YY1), respectively. We observed a significant increase in YY1 staining in tumor and PIN samples compared to non-malignant samples (morphologically normal and BPH tissues, p<0.0001; Table II). As a group, 82% of tumor-containing and 76% of PIN-containing spots showed moderate to strong nuclear staining, whereas only 57% of normal and 34% of BPH tissue spots displayed equivalent nuclear staining (Fig. 5A).

Table II. Association of benign[a] and neoplastic[b] tissue groups by nuclear or cytoplasmic YY1 expression variables (per spot comparison; n=1,061).

YY1 expression scoring method	Benign versus neoplastic expression[c]	
	χ^2	P-value
Nuclear intensity	107.5	<0.0001
Nuclear positivity[d]	216.3	<0.0001
Cytoplasmic intensity	199.6	<0.0001
Cytoplasmic positivity	34.6	<0.0001

[a]n=333 array spots; [b]n=728 array spots; [c]Kuskal-Wallis test; [d]Variable measure used for clinical outcome studies.

Interestingly, the proportion of tumor spots displaying moderate to strong staining increased abruptly with grade Gleason grade ≥3 (graph not shown). Compared to Gleason grades 3, 4 and 5, for which 84, 87 and 79% of tissues stained at that level, only 65% of low-grade tumor spots (Gleason grades 1 and 2) stained the same (grade 1-2 versus grade ≥3, p<0.0001).

The frequency of cells with nuclear YY1 staining followed the same trend as was seen with staining intensity; a higher proportion of tumor and PIN tissue spots stained with higher frequency (50-100% category) compared to normal and BPH (Fig. 5B; p<0.0001). In summary, in the neoplastic lesions, there is a concomitant increase in both the amount of nuclear YY1 expression per cell and in the proportion of cells with nuclear staining.

Cytoplasmic YY1 staining. In addition to nuclear staining, we were somewhat surprised to see tissue samples with a relatively high cytoplasmic expression of YY1 (Fig. 4G). This staining appeared specific as it was concentration-dependent, present in some, but not all cells, and inhibited by the immunizing peptide (data not shown). Fig. 5C and D show the staining distribution and frequency of cytoplasmic YY1 staining in the TMA. Similar to nuclear YY1 staining, there was a higher intensity of cytoplasmic YY1-staining cells in PIN and tumor tissue spots compared to benign histologies (p<0.0001; Table II). The majority of all tissue spot histology categories showed ≥50% of the cells staining positively for cytoplasmic YY1 (Fig. 5D). The cut-off of 50% was discussed above.

Interestingly, there was a strong correlation between cytoplasmic and nuclear YY1 staining (i.e., the trends observed with regard to histologies or outcomes were similar for either cytoplasmic or nuclear staining). Thus, we show only one set of data, nuclear YY1 staining, below.

YY1 expression and cancer recurrence. We next examined whether nuclear YY1 expression was associated with tumor
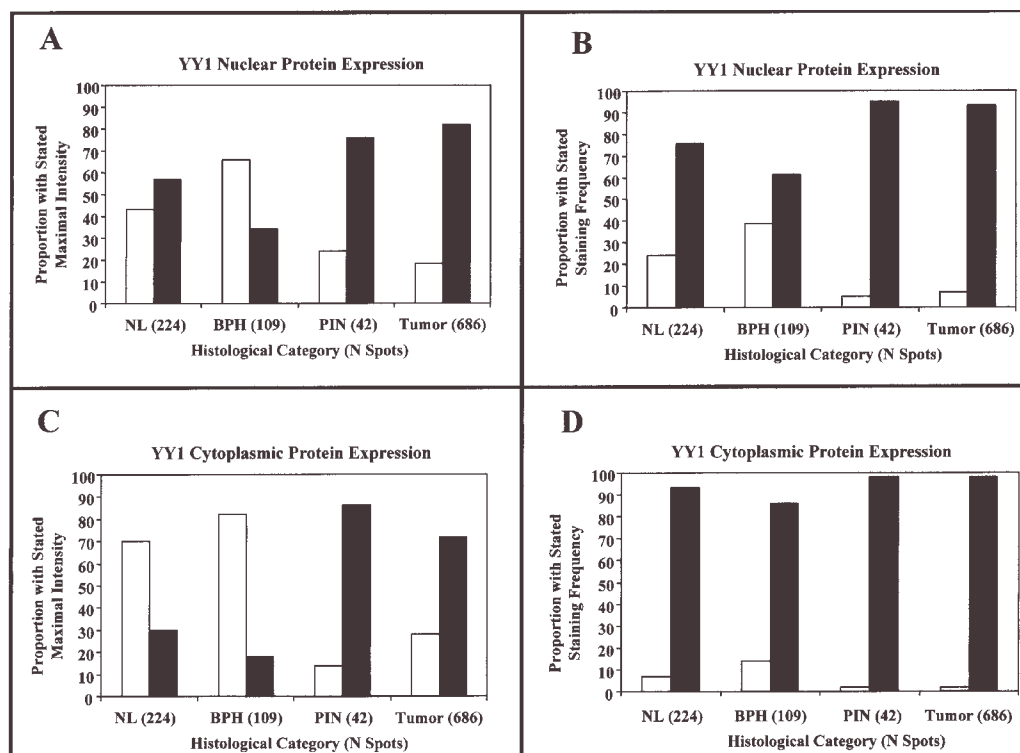

Figure 5. YY1 protein expression distribution on the prostate TMA stratified by histological category. Shown are the proportional distributions of YY1 protein staining by immunohistochemistry with attention to the maximal nuclear and cytoplasmic staining intensity, (A and C, respectively), and the total proportion of nuclear and cytoplasmic positivity at any intensity (B and D, respectively) of the target cells of the appropriate histologic category of each spot. Twelve informative spots representing metastases are not included here. Staining pattern for (A) and (C): □, negative to weak; ■, moderate to strong. Staining frequency for (B) and (D): □, 0-49% of cells; ■, 50-100% of cells.

Table III. Cox proportional hazards analyses.[a]

Variable	Univariate (all patients[b]) n=190	Multivariate (all patients[c]) n=169		Univariate (low Gleason[d]) n=112	Multivariate (low Gleason[e]) n=102		Univariate (high Gleason[f]) n=78	Multivariate (high Gleason[g]) n=67	
		Continuous	Dichotomized		Continuous	Dichotomized		Continuous	Dichotomized
Seminal vesicle invasion (Stage = pT3b)	**<0.0001** 4.61 (2.73-7.76)	**0.0015** 2.73 (1.47-5.07)	**0.0016** 2.71 (1.46-5.03)	**0.0043** 6.08 (1.76-21.04)	**0.0062** 6.69 (1.71-26.15)	**0.0093** 6.23 (1.57-24.74)	0.0036 2.45 (1.34-4.48)	0.0089 2.46 (1.25-4.81)	0.0099 2.43 (1.24-4.76)
Gleason score >7	**<0.0001** 3.96 (2.35-6.67)	**0.0036** 2.67 (1.38-5.18)	**0.0032** 2.70 (1.39-5.23)	NA	NA	NA	NA	NA	NA
Preoperative PSA	**0.0008** 1.03 (1.01-1.04)[h]	0.29 1.01 (0.99-1.03)	0.30 1.01 (0.99-1.03)	**0.027** 1.04 (1.01-1.08)[i]	0.054 1.03 (1.00-1.07)	0.071 1.03 (0.99-1.07)	0.37 1.01 (0.99-1.03)[j]	0.53 1.01 (0.99-1.03)	0.58 1.01 (0.99-1.03)
Capsular invasion	**0.0015** 1.82 (1.26-2.64)	**0.0063** 1.93 (1.20-3.09)	**0.0055** 1.95 (1.22-3.13)	**0.0067** 2.41 (1.28-4.56)	**0.0044** 3.52 (1.48-8.34)	**0.0041** 3.48 (1.49-8.16)	0.53 1.18 (0.71-1.97)	0.42 1.28 (0.70-2.35)	0.39 1.31 (0.71-2.41)
YY1 nuclear staining (continuous)[k]	**0.011** 0.99 (0.98-0.99)	**0.011** 0.99 (0.98-0.99)	NA	**0.031** 0.99 (0.97-0.99)	**0.015** 0.98 (0.97-0.99)	NA	0.28 0.99 (0.99-1.00)	0.27 0.99 (0.98-1.00)	NA
YY1 nuclear staining (>50%, dichotomized)[l]	**0.016** 0.53 (0.31-0.89)	NA	**0.012** 0.47 (0.27-0.85)	0.091 0.45 (0.18-1.13)	NA	0.052 0.35 (0.12-1.01)	0.17 0.64 (0.34-1.21)	NA	0.17 0.60 (0.29-1.25)

[a]P-value; Hazard ratio; (95% confidence interval) provided. [b]66% of cases are censored. [c]69% of cases are censored. [d]Gleason score 2-6; 81% of cases are censored. [e]Gleason score 2-6; 83% of cases are censored. [f]Gleason score 7-9, (no Gleason score 10 cases are present); 44% of cases are censored. [g]Gleason score 7-9, (no Gleason score 10 cases are present); 45% of cases are censored. [h]n=169. [i]n=102. [j]n=67. [k]Pooled cases with minimum YY1 positive staining. [l]Pooled cases with minimum YY1 positive staining dichotomized: ≤50% (n=42); > 50% (n=148).

recurrence following prostatectomy. In this study, recurrence was defined as the postoperative presence of serum PSA. Recurrence data were available for 190 patient cases from patients in the tissue array. Array spots from a given case were pooled as previously described (26) and analyzed for the percentage of invasive malignant cells expressing nuclear YY1 (see Materials and methods). Results were examined both as a continuous and dichotomized population. The most significant results were obtained when we considered cases that contained the least percentage of YY1 staining (defined in Materials and methods). As continuous variable, YY1 was a significant predictor of recurrence in both univariate and multivariate Cox proportional hazards models (p=0.011; 0.99; 95% CI 0.98-0. 99 for both models; Table III).

We further analyzed the dichotomized cases. As discussed above, cases were divided based on spots in which ≤50% of the cells expressed nuclear YY1 (referred to as 'YY1-low' cases) or in which >50% of cells expressed nuclear YY1 (referred to as 'YY1-high' cases). These two distinct patient groups did not associate with traditional clinicopathological covariates (Table I). Surprisingly, patient's whose tissue had
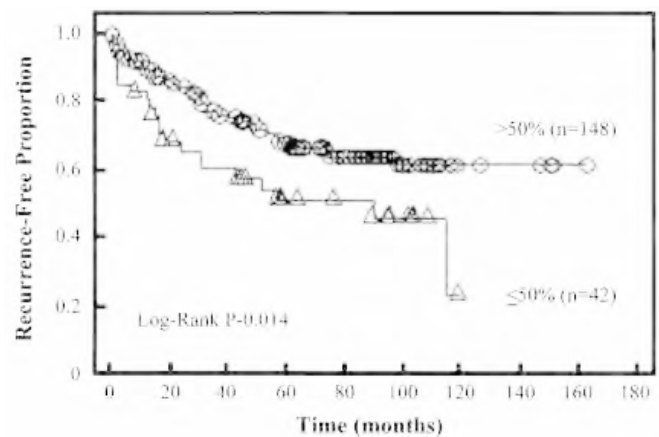
Figure 6. Kaplan-Meier curve for time to recurrence. Kaplan-Meier curve for time to tumor recurrence stratified by nuclear YY1 protein expression status (n=190 patients total). The upper and lower curves contain groups of individuals whose tumors demonstrated a minimal staining frequency of >50 or ≤50%, respectively. Censored patients are indicated as open circles (70% censored) or triangles (50% censored). A low nuclear YY1 expression phenotype is significantly associated with a higher risk to develop recurrent disease.

higher percentage of YY1 staining (the YY1-high cases) had a longer time to cancer recurrence compared with the YY1-low cases (Table III; p=0.016; hazard ratio 0.53; 95% CI 0.31-0.89, univariate). Fig. 6 shows a Kaplan-Meier estimate of recurrence (cancer-free) time for all 190 patients (log-rank p=0.014). The median recurrence-free time was 90 months in YY1-low cases, compared to >163 months in YY1-high cases. Moreover, only 30% of the YY1-high cases had a tumor recurrence (70% were censored), compared to 50% of the YY1-low cases (50% censored cases).

The dichotomized YY1 groups proved to be independent predictors of recurrence in multivariate Cox proportional hazards model including all 190 patients (p=0.012, hazard ratio 0.47, 95% CI 0.27-0.85). The associations was not significant when patients were substratified by tumor grade (Table III).

In summary, while the majority of prostate tumors examined expressed ample YY1, there was an increase in the time for disease recurrence if an individual's tumor had regions of lower YY1 expression.

Discussion

In this study we have examined the expression pattern of YY1 in normal and malignant prostate tissue using a prostate tissue microarray. The genesis of this investigation stemmed from results using a cell culture system which demonstrated that YY1 expression contributed to the inhibition of Fas expression and thus decreased sensitivity to Fas-mediated apoptosis (13). Based on these initial studies, we started to examine the YY1 expression level and pattern in human normal and malignant prostatic tissue. To do this, we took advantage of tissue microarray technology, constructing an array of case material from 246 prostatectomies. This tissue microarray contains all relevant histologies/pathologies linked to outcomes data when available, thus allowing us to examine YY1 expression pattern in a relatively large cohort of patients. When we examined YY1 protein expression levels using immunohistochemistry, we observed an increase in nuclear and cytoplasmic YY1 expression in tumor and PIN samples compared to histologically normal or BPH tissues. This was the case for both staining intensity and for the percentage of cells that stained positively. Notably, this represents the first association of YY1 expression with prostate cancer progression.

YY1 transcription regulation. While YY1 has been described as a context-specific positive or negative regulator of transcription, the exact mechanism of action of YY1 is currently unknown (14,15). Proposed models for YY1 function include context-specific activation or repression, interaction with other transcription factors or modulating proteins [e.g., transcription factor IIB (TFIIB), Sp1, c-myc, Rb, the notch receptor, YY1AP, p300 and CBP], regulation of p53 ubiquitinylation, and/or chromatin modification (e.g., histone acetylation or deacetylation) (14-16,28-37). Relevant to processes such as inflammation, immune responses, and tumor initiation/ progression, and cell cycle progression, YY1 can modulate the expression of genes such as c-myc (38), c-fos (39,40), p53 (16,41), human IFN-γ gene (17,18), Fas (13), IL-3 (20), IL-4 (42), GM-CSF (17,20), IFN-ß (43), histone (44) and

CCR5 (45). Whether these and/or other gene(s) are primarily influenced or modulated by YY1 in prostatic epithelial cells *in vivo* remains to be determined.

Of particular note is the recently described role of YY1 in regulating levels of the tumor suppressor p53 by affecting its ubiquitination by Mdm2 (16) and/or its interaction with p300 (46). In this study, increased expression of YY1 promoted the ubiquitination and resultant steady-state reduced expression of p53. Alterations in p53 with the subsequent loss of wild-type function is one of the most common events in human cancers (47-50). Although the data are still somewhat ambiguous, as much as 94% of prostate cancer cases have some alteration in p53 (51-53). There are reports that indicate that alterations in p53 can be both an early and/or a late event in prostate cancer development (52-54). It is an intriguing possibility that one of the consequences of changes in YY1 expression and/or localization is disruption of p53 or p53-dependent pathways thus contributing to the malignant process.

Although in our *in vitro* studies YY1 was inversely associated with Fas expression, there was no obvious correlation *in vivo* by immunohistochemical analysis (data not shown). There are several possible explanations for this. First, it is possible that this merely represents a technical limitation of immunohistochemistry to simultaneously detect localized changes in Fas and YY1. Second, *in vivo*, YY1 activity as opposed to YY1 expression levels, might be the determinant of Fas expression. YY1 activity would not be accurately measured by the assays utilized in this study. Finally, potential discordances between *in vitro* and *in vivo* observations could reflect differences in cellular milieu. The *in vitro* system modeled IFN-γ-induced up-regulation of Fas; this up-regulation occurred due to inactivation of YY1 by NO (13). These conditions may not exist in these clinical samples where the relative abundance and influence of IFN-γ are unknown. Nevertheless, we continue to explore the interplay between YY1, Fas, NO and IFN-γ in this as well as other systems.

YY1 expression pattern in malignant prostate tissue. In non-malignant prostatic epithelium, YY1 was present predominantly in the nucleus of glandular epithelium and basal cells consistent with its activity in transcription regulation. Interestingly, there was often staining in the cytoplasm of these cells as well; cytoplasmic became more pronounced in PIN and more malignant cells. For example, greater than 95% of the malignant samples examined displayed significant cytoplasmic staining (score of 2-3) (Fig. 5). Recently, Palko *et al* reported that YY1 transits from the cytoplasm to nucleus at various stages of the cell cycle (55). Specifically, YY1 was localized primarily in the nucleus during late G1-early S phase, but primarily distributed in the cytoplasm during G1 and late S phase (55). A limited number of other groups have similarly observed localization of YY1 in both the cytoplasm and nucleus in various model systems (56,57). In this light, the observation in our study that the majority of tumor samples contained malignant cells with relatively high levels of both nuclear and cytoplasmic staining is intriguing. The mechanism that regulates the migration of YY1 from the cytoplasm to the nucleus is unknown but may be dependent on nuclear localization signals, specific protein interactions such as observed with IκB,

and/or intracellular shuttling proteins. In regard to the later mechanism, the nuclear/nucleolar shuttle protein nucleophosmin (B23) is known to bind to YY1 (58,59). It is interesting to consider that the high correlation of expression of YY1 in both nuclear and cytoplasmic compartments in malignant cells may contribute to dysfunction of YY1 activity. Such a mechanism awaits further definition.

Regions of low YY1 expression predict a poorer outcome. The results that we obtained when examining the potential relationship between YY1 expression/distribution and tumor recurrence were interesting and somewhat surprising. Rather than high or more abundant levels of YY1 being a predictor for more rapid tumor recurrence, tumors that were heterogeneous and displayed more regions of minimal YY1 staining correlated with poorer outcome (Fig. 6). Tumor recurrence is defined as an increase in PSA levels following a prostatectomy thus indicating the presence of metastatic tumor cells. Although formal proof is required, these data raise the possibility that decreased YY1 expression may enhance the survival of metastatic prostate cancer cells. Although there are numerous potential reasons for why decreased YY1 expression could provide a survival advantage for metastatic cells, it is conceivable that expression or repression of a new repertoire of genes would be required to survive in a new milieu. Such a mechanism requires further examination.

Our results are similar to those observed by Hofer *et al*, for the expression of metastasis-associated gene 1 (MTA1) in prostate cancer (60). MTA1 was originally identified from differential screening of the rat mammary adenocarcinoma non-metastatic cell line, MTC.4, versus the metastatic cell line, MTLn3; MTA1 was overexpressed in the latter (61,62). Subsequently, MTA1 was found to be overexpressed in invasive lesions of various human cancers (60,63-67). Hofer *et al*, observed that MTA1 was expressed at highest levels in metastatic prostate cells versus either non-malignant or clinically localized malignant cells (60). Moreover, they found that higher expression levels of MTA1 correlated with a longer PSA-fee period following prostatectomy, whereas negative or weak MTA1 expression correlated with an increased time to tumor recurrence (60). While the exact function of the MTA protein family has yet to be determined, there is recent evidence suggesting that MTA1 is part of the nucleosome remodeling histone deacetylation (NuRD) complex (68). That decreased expression of both MTA1 and YY1 is correlated with a more rapid time to tumor recurrence, and that both proteins share histone deacetylation function may be more than mere coincidence. Studies are currently underway to test this hypothesis.

In conclusion, YY1 joins an expanding list of proteins whose expression or activity is altered during the course of prostate cancer progression (69,70). The general increase in YY1 expression in malignant compared to benign cells, as well as the associated increase survival in patients with tumors displaying decreased YY1 expression is intriguing. We predict that YY1 and/or proteins present in YY1-dependent pathways, will become part of a profile of proteins that may be useful diagnostic or prognostic tools as well as potential therapeutic targets.

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REVIEW

Transcription factor YY1: structure, function, and therapeutic implications in cancer biology

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The ubiquitous transcription factor Yin Yang 1 (YY1) is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation. YY1 exerts its effects on genes involved in these processes via its ability to initiate, activate, or repress transcription depending upon the context in which it binds. Mechanisms of action include direct activation or repression, indirect activation or repression via cofactor recruitment, or activation or repression by disruption of binding sites or conformational DNA changes. YY1 activity is regulated by transcription factors and cytoplasmic proteins that have been shown to abrogate or completely inhibit YY1-mediated activation or repression; however, these mechanisms have not yet been fully elucidated. Since expression and function of YY1 are known to be intimately associated with progression through phases of the cell cycle, the physiologic significance of YY1 activity has recently been applied to models of tumor biology. The majority of the data are consistent with the hypothesis that YY1 overexpression and/or activation is associated with unchecked cellular proliferation, resistance to apoptotic stimuli, tumorigenesis and metastatic potential. Studies involving hematopoetic tumors, epithelial-based tumors, endocrine organ malignancies, hepatocellular carcinoma, and retinoblastoma support this hypothesis. Molecular mechanisms that have been investigated include YY1-mediated downregulation of p53 activity, interference with poly-ADP-ribose polymerase, alteration in c-myc and nuclear factor-kappa B (NF-κB) expression, regulation of death genes and gene products, and differential YY1 binding in the presence of inflammatory mediators. Further, recent findings implicate YY1 in the regulation of tumor cell resistance to chemotherapeutics and immune-mediated apoptotic stimuli. Taken together, these findings provide strong support of the hypothesis that YY1, in addition to its regulatory roles in normal biologic processes, may possess the potential to act as an initiator of tumorigenesis and may thus serve as both a diagnostic and prognostic tumor marker; furthermore, it may provide an effective target for antitumor chemotherapy and/or immunotherapy.

Introduction

The critical role of transcription factors in the regulation of cell function has been undoubtedly established; their tasks in activation, repression, and/or modification of gene expression are necessary and required for growth, development, and differentiation (Shi et al., 1997). Several lines of evidences have demonstrated deleterious outcomes when transcription factors become dysfunctionally activated or inactivated, leading to cellular malfunction, instability, and in some cases, tumorigenesis. Yin Yang 1 (YY1) is one such ubiquitous transcription factor. It is important to divulge how complex factors such as YY1 function in diverse biological processes and ultimately shape the growth and viability of eukaryotic cells. Thus, the biology of YY1 and its fundamental properties that initiate proper cellular development and the recently expanded potential role for YY1 in cancer biology, specifically the regulation of and resistance to cancer therapeutics, will be highlighted in this review.

YY1 discovery

YY1 is a ubiquitous and multifunctional zinc-finger transcription factor (also known as δ, NF-E1, UCRBP, and CF1) member of the Polycomb Group protein family, a group of homeobox gene receptors that play critical roles in hematopoiesis and cell cycle control. YY1 was initially cloned and characterized simultaneously by two independent groups, Shi et al. (1991) and Park and Atchison (1991) who were inspired by the original observation by Berns and Bohenzky (1987) and Chang et al. (1989). While investigating the adeno-associated virus (AAV) P5 promoter region and its

activation by E1A gene products, using systematic deletion analysis of the P5 promoter, Chang et al. (1989) identified two elements associated with basal and E1A-induced P5 activity: (1) the R1–R2 region (P5-60 site), a tandem repeat sequence of 10 base pairs, and (2) a binding site for the major late transcription factor (MLTF). Both elements had a negative effect in the absence of E1A oncoprotein, but converted to transcriptional activators in its presence. They theorized that the two trans-activators acted in concert to stimulate the P5 promoter and induce transcriptional activation in the presence of E1A. Noteworthy, simultaneous deletion of both elements reduced P5 promoter activity 25-fold, raising the possibility of the presence of the dual-acting transcriptional factor YY1 (Chang et al., 1989). A subsequent study reported by Shi et al. (1991) once again identified two cellular protein complexes interacting with the P5-60 site of AAV P5 promoter. Band shift assays using a 22-base pair oligonucleotide containing the P5-60 element, detected the formation of a protein complex that was competed out by addition of excess unlabeled P5-60 oligonucleotide but not by the oligonucleotide (P5ML) containing the binding site for the MLTF. The major protein component of this complex was termed YY1. A second P5-60-specific binding protein (termed factor 2) was identified but masked by a co-migrating complex formed by a nonspecific DNA-binding activity in HeLa cells. Shi et al. (1991) formally named the factor YY1. During the preparation of the manuscript, Shi et al. (1991) learned that YY1 had been cloned by two additional laboratories. Park and Atchison (1991) have identified and cloned the protein, which they termed NF-E1, based on its ability to bind within the Igκ 3′ enhancer (Park and Atchison, 1991). Hariharan et al. (1991) identified and cloned the protein, which they termed δ, based on its ability to bind to sequence elements downstream of the transcriptional start sites in the ribosomal protein L30 and L32 genes. Subsequently, YY1 has been identified in other species and has been assigned alternate nomenclature by other authors, including UCRBP (Flanagan et al., 1992), nuclear matrix protein NMP1 (Guo et al., 1995), and common factor 1 (Thomas and Seto, 1999) (Table 1).

YY1 structure

Chromosomal localization and molecular structure. A purified YY1 genomic DNA probe was used in FISH analysis to map the location of the YY1 gene to the telomere region of human chromosome 14 at segment q32.2 (Yao et al., 1998). The YY1 gene consists of five highly conserved exons encoding a protein of 414 amino acids in length, and an estimated molecular weight of 44 kDa. However, due to the structure of the protein, SDS–polyacrylamide gel analysis reveals its weight to be 68 kDa (Shi et al., 1997). Figure 1 illustrates significant similarities between human and mouse YY1 nucleotide sequences. According to AceView database (http://www.ncbi.nih.gov/IEB/Research/Acembly/index.html), the sequence of the YY1 gene is supported by 850 sequences from 781 cDNA (accessed November 2004). The human YY1 gene produces eight different transcripts (a, b, c, d, e, f, g, and h) generated by alternative splicing, encoding eight different putative protein isoforms (three complete, three COOH-complete, and two partial). The functional significance of these isoforms remains elusive. There are two alternative promoters. Different transcripts differ by truncation of the 5′ end, truncation of the 3′ end, presence or absence of four cassette exons, and different boundaries on common exons due to variable splicing of an internal intron (Figure 2).

Biochemical and crystal structure. The YY1 protein contains four C_2H_2-type zinc-finger motifs with two specific domains that characterize its function as an activator or repressor. Analysis of GAL4 fusion protein revealed repression of transcription by the C-terminus domain (aa 298–397) (Shi et al., 1991, 1997) using a chloramphenicol acetyl transferase (CAT)-based reporter system driven by a promoter rich in GAL-4-binding sites. Two other domains contributing to its repression include sequences within the zinc-finger motifs and a glycine-rich residue between amino acids 157 and 201. The N-terminus region (aa 43–53), however, acts as a potent activation domain (Shi et al., 1997; Nguyen et al., 2004). This region is followed by a glycine-rich domain

Table 1 YY1 aliases by species

Species	Aliases	Chromosome	References
Homo sapiens	Delta (δ), nuclear factor E1 (NF-E1), upstream conserved region binding protein (UCRBP), common factor 1 (CF1), nuclear matrix protein 1 (NMP-1), nuclear factor D (NF-D), F-ACT1	14q32	Park and Atchison (1991), Chen et al. (1992), Lee et al. (1992), Riggs et al. (1993), Martelli et al. (1996) and Guo et al. (1995)
Mus musculus	NF-E1, UCRBP transcription factor, delta transcription factor	12	Park and Atchison (1991), Chen et al. (1992) and Satyamoorthy et al. (1993)
Danio rerio	fa16g07, fb59g10, wu:fa16g07, wu:fb59g10	LG 17	Park and Atchison (1991), Chen et al. (1992) and Satyamoorthy et al. (1993)
Rattus norvegicus	NF-E1, UCRBP	6q32	Park and Atchison (1991), Chen et al. (1992) and Satyamoorthy et al. (1993)
Xenopus laevis	yy1-A-prov, FIII	Not described	Pisaneschi et al. (1994)

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   1 ATGGCCTCGGGCGACACCCTCTACATCGCCACGGACGGCTCGGAGATGCCGGCCGAGATC
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   1 ATGGCCTCGGGCGACACCCTCTACATCGCCACGGACGGCTCGGAGATGCCGGCCGAGATC

  61 GTGGAGCTGCACGAGATCGAGGTGGAGACCATCCCGGTGGAGACCATCGAGACCACAGTG
     ||||||||||  ||||||||||||||||||||||||||||||||||||||||||||| |||
  61 GTGGAGCTGCATGAGATCGAGGTGGAGACCATCCCGGTGGAGACCATCGAGACCACGGTG

 121 GTGGGCGAGGAGGAGGAGGAGGACGACGACGACGAGGACGGCGGCGGTGGCGACCA---C
     ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| |
 121 GTGGGCGAGGAGGAGGAGGAGGAGGACGACGACGACGAGGACGGCGGCGGCGGCGACCACGGC

 178 GGCGGCGGGGGCGGCCACGGGCACGCCGGCCACCAC---CACCACCACCATCACCACCAC
     |||||||||||||||||||||||||||||||||||   ||||||||||| |||||||||
 181 GGCGGCGGGGGCGGCCACGGGCACGCCGGCCACCACCATCACCACCACCACCACCACCAC

 235 CACCACCCGCCCATGATCGCTCTGCAGCCGCTGGTCACCGACGACCCGACCCAGGTGCAC
     ||||||||||||||||||||| ||||||||||||| || ||||||||||||||| |||||
 241 CACCACCCGCCCATGATCGCGCTGCAGCCGCTGGTGACGGACGACCCGACCCAAGTGCAC

 295 CACCACCAGGAGGTGATCCTGGTGCAGACGCGCGAGGAGGTGGTGGGCGGCGACGACTCG
     |||||||||||||||||||||||||||||||||||||||||||| ||||| |||||||||
 301 CACCACCAGGAGGTGATCCTGGTGCAGACGCGCGAGGAGGTGGTCGGCGGGGACGACTCG

 355 GACGGGCTGCGCGCCGAGGACGGCTTCGAGGATCAGATTCTCATCCCGGTGCCCGCGCCG
     ||||||||||||||||||||||||||||||| ||||| |||||||||||||||||||||
 361 GACGGGCTGCGCGCCGAGGACGGCTTCGAGGACCAGATCCTCATCCCGGTGCCCGCGCCG

 415 GCCGGCGGCGACGACGACTACATTGAACAAACGCTGGTCACCGTGGCGGCGGCCGGCAAG
     |||||||||||||||||||||| ||  || |||||||||||||||||||||||||||||
 421 GCCGGCGGCGACGACGACTACATAGAGCAGACGCTGGTCACCGTGGCGGCGGCCGGCAAG

 475 AGCGGCGGCGGCGGCTCGTCGTCGTCGGGGAGGCGGCCGCGTCAAGAAGGGCGGCGGCAAG
     ||||||||||||      | ||  |||||||||||||| ||||||||||||||||||||
 481 AGCGGCGGCGG------GGCCTCGTCGGGCGGCGGTCGCGTGAAGAAGGGCGGCGGCAAG

 535 AAGAGCGGCAAGAAGAGTTACCTCAGCGGCGGGGCCGGCGCGGCGGGGCGGCGGCGGCGCC
     |||||||||||||||||||||| |  |||||||||||||||||||||| |||||||||||
 535 AAGAGCGGCAAGAAGAGTTACCTGGGCGGCGGGGCCGGCGCGGCGGGCGGCGGCGGCGCC

 595 GACCCGGGCAACAAGAAGTGGGAGCAGAAGCAGGTGCAGATCAAGACCCTGGAGGGCGAG
     ||||||| |  |||||||||||||||||||||||||||||||||||||||||||||||||
 595 GACCCGGGGAATAAGAAGTGGGAGCAGAAGCAGGTGCAGATCAAGACCCTGGAGGGCGAG

 655 TTCTCGGTCACCATGTGGTCCTCAGATGAAAAAAAAGATATTGACCATGAGACAGTGGTT
     |||||||||||||||||||||||| |||||||||||||||||||||||||| ||||||||
 655 TTCTCGGTCACCATGTGGTCCTCGGATGAAAAAAAAGATATTGACCATGAAACAGTGGTT

 715 GAAGAACAGATCATTGGAGAGAACTCACCTCCTGATTATTCAGAATATATGACAGGAAAG
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 715 GAAGAGCAGATCATTGGAGAGAACTCACCTCCTGATTATTCTGAATATATGACAGGCAAG

 775 AAACTTCCTCCTGGAGGAATACCTGGCATTGACCTCTCAGATCCCAAACAACTGGCAGAA
     ||||| |||||||||||| |||||||||||||||||||||| || || |||||||||||
 775 AAACTCCCTCCTGGAGGGATACCTGGCATTGACCTCTCAGACCCTAAGCAACTGGCAGAA

 835 TTTGCTAGAATGAAGCCAAGAAAAATTAAAGAAGATGATGCTCCAAGAACAATAGCTTGC
     ||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
 835 TTTGCCGAATGAAGCCAAGAAAAATTAAAGAAGATGATGCTCCAAGAACAATAGCTTGC

 895 CCTCATAAAGGCTGCACAAAGATGTTCAGGGATAACTCGGCCATGAGAAAACATCTGCAC
     ||||||||||||||||||||||||||||||||||||||| || |||||||| |||||||
 895 CCTCATAAAGGCTGCACAAAGATGTTCAGGGATAACTCTGCTATGAGAAAGCATCTGCAC

 955 ACCCACGGTCCCAGAGTCCACGTCTGTGCAGAATGTGGCAAAGCTTTTGTTGAGAGTTCA
     ||||||||||||||||||||||||||||||||| ||||||||||| || |||||||| ||
 955 ACCCACGGTCCCAGAGTCCACGTCTGTGCAGAGTGTGGCAAAGCGTTCGTTGAGAGCTCA

1015 AAACTAAAACGACACCAACTGGTTCATACTGGAGAGAAGCCCTTTCAGTGCACGTTCGAA
     || |||||||||||||| ||||||||||||||||||| |||||||||||||||| |||||
1015 AAGCTAAAACGACACCAGCTGGTTCATACTGGAGAAAAGCCCTTTCAGTGCACATTCGAA

1075 GGCTGTGGGAAACGCTTTTCACTGGACTTCAATTTGCGCACACATGTGCGAATCCATACC
     ||||| ||||| || |||||||||||||||||||||||||||||||||||||||||||||
1075 GGCTGCGGGAAGGCGTTTTCACTGGACTTCAATTTGCGCACACATGTGCGAATCCATACC

1135 GGAGACAGGCCCTATGTGTGCCCCTTCGATGGTTGTAATAAGAAGTTTGCTCAGTCAACT
     |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
1135 GGAGACAGGCCCTATGTGTGCCCCTTCGACGGTTGTAATAAGAAGTTTGCTCAGTCAACT

1195 AACCTGAAATCTCACATCTTAACACATGCTAAGGCCAAAAACAACCA
     |||||||||||||||||||||||||||| ||||| ||||||||||||
1195 AACCTGAAATCTCACATCTTAACACACGCTAAAGCCAAAAACAACCA
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Figure 1 YY1 DNA Sequence homology. Sequence comparison of the main open reading frame of the human (M77698) YY1 gene (Top sequence) and the mouse (M73963) YY1 gene (Bottom sequence) showing 94.9% of identity and similarity determined by the Smith-Waterman alignment of nucleic acids. Both coding regions encode for a putative protein of 414 amino acids with a predicted molecular weight of approximately 44 kDa.

and 11 consecutive histidine residues (aa 70–80). The role of this sequence remains elusive (Shi et al., 1997). The cocrystal structure of YY1 is shown in Figure 3 (Houbaviy et al., 1996).

Family/homology

Family. The YY1 sequence homology to the *Drosophila* Krüppel protein, a peptide initially described in 1984 and shown to be necessary for embryogenesis and normal morphology via transcriptional activation and repression, identifies it as a member of the GLI-Krüppel gene family (Wieschaus et al., 1984; Shi et al., 1991, 1997).

Homology. The fundamental role of YY1 in development and cellular propagation is supported by studies demonstrating mammalian cDNA encoding a YY1-binding protein possessing sequence homology with the yeast transcription factor reduced potassium dependence 3 protein (RPD3) (Yang et al., 1996). Thus, critical sequences reveal a high degree of interspecies homology for this transcriptionally active gene. More recent DNA and amino acid sequence database analyses show striking similarities in structure and function of YY1 to a newly discovered sister protein, Yin Yang 2 (YY2) (Nguyen et al., 2004). Deletion analysis reveals that YY2, like YY1, contains both activation and repression domains (N-terminus and C-terminus, respectively). In fact, it is heavily involved in gene regulation controlled by YY1. YY2 has been shown to interact with most, but not all promoter-binding sites associated with YY1 and has an almost identical YY1-like cDNA with slight nucleotide differences. The aa 256–365 sequence reveals an 86% homology to the zinc fingers of YY1 and 62% homology to the spacer regions. (Nguyen et al., 2004). The functions of these regions may have possible implications for the activities of YY1 and YY2, but are yet to be elucidated.

Role of YY1 in transcriptional regulation

Activation versus *repression*

By tethering to DNA promoters, YY1 regulates a variety of cellular and viral genes (Donohoe et al., 1999; Nguyen et al., 2004). What distinguishes this protein from other transcription factors is its ability to not only initiate transcription but also regulate it through activation or repression. Studies have repeatedly shown the association and modulation of YY1 by adenovirus-derived E1A, a protein that activates the AAV P5 promoter (Chang et al., 1989). The presence of E1A induces YY1-mediated activation of transcription. In its absence, the role of YY1 is reversed, converting to a transcriptional repressor (Shi et al., 1997); hence the name Yin Yang 1.

To clarify the process by which activation is favored in the presence of E1A, but switched to repression in its absence, studies were designed to test its functional status by masking and/or exposing the binding sites of
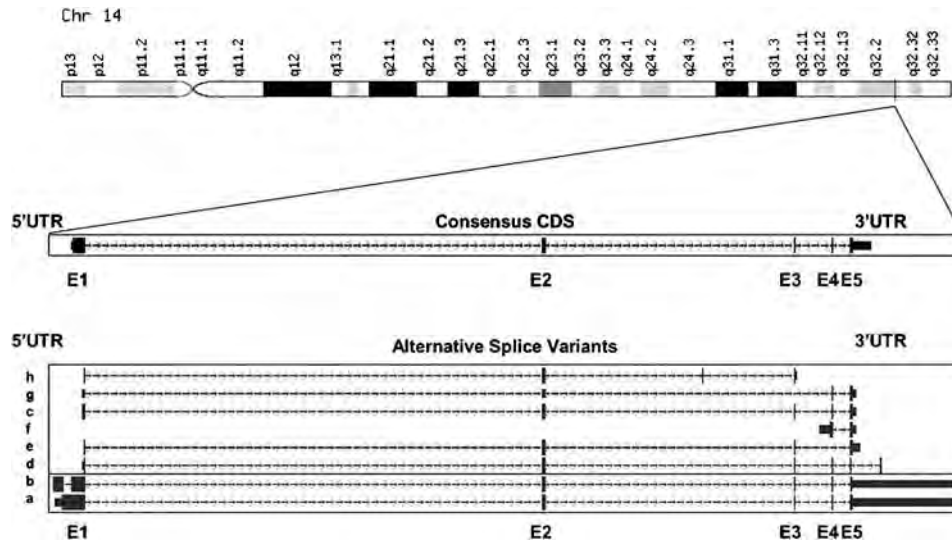
Figure 2 Gene organization and alternative splice variants of the YY1 gene. This figure schematically illustrates the localization of YY1 to chromosome 14. Eight different transcripts (**a–h**) generated by alternative slicing, encode eight different putative protein motifs.

Figure 3 YY1 Cocrystal structure. The cocrystal structure of YY1 is shown (Houbaviy *et al*., 1996, Research Collaboratory for Structural Bioinformatics Protein Data Bank http://www.pdb.org, accessed January 2005). The protein contains four C_2H_2-type zinc-finger motifs with two specific domains that characterize its function as an activator or repressor. Transcriptional repression is known to occur at the C-terminus (aa 298–397) directed by a promoter rich in GAL-4-binding sites. The N-terminus (aa 43–53), acts as a potent activation domain. Evidence that the zinc-fingers and glycine-rich regions of YY1 are instrumental in YY1 repression has been provided by deletional experiments of both regions, which render the protein incapable of transcriptional repression.

YY1. In the absence of E1A, the AAV virus fails to undergo transcription, most likely due to YY1 binding to the P5 promoter (Shi *et al*., 1991). Mechanisms that have been proposed to explain this phenomenon include the possibility of a conformational change in YY1 through covalent modification, or a direct interaction between YY1 and an E1A-type accessory protein. The mechanisms by which this occurs remain unclear.

Embryogenesis, growth, and differentiation
YY1 also plays pivotal roles in mammalian biological processes. Donohoe *et al*. (1999) examined genotyped mouse embryos at different gestational stages. Mouse embryos made homozygous for the mutated YY1 allele did not survive; after uterine implantation they failed to develop beyond the blastula stage. YY1 heterozygotes survived, but displayed significant growth retardation and neurological defects, suggesting the significance of functional YY1 activity during later stages of mouse embryogenesis. Similar results were seen during the development of the African clawed frog, *Xenopus laevis* (Morgan *et al*., 2004).

Kurisaki *et al*. (2003) recently identified YY1 as a nuclear factor that interacts with mothers against dPP (MAD) and Mad-related (Smad) complexes, the principal signaling proteins of intracellular factors including transforming growth factor beta (TGF-β) and bone morphogenic protein (BMP), both of which are responsible for cell growth and differentiation. YY1 was found to interact with and repress Smad-specific transcriptional activity, suggesting its essential function in cell differentiation stimulated by TGF-β1 and other nuclear factors.

Proliferation and response to genotoxic stimuli
The ubiquitous presence of YY1 suggests important roles for cellular stability and normal functioning. YY1 has recently been found to activate DNA repair. Studies have shown an enhanced stimulation of poly (ADP-ribose) polymerase-1 (PARP-1) in HeLa cells

transfected to overexpress YY1 after exposure to methyl-*N*-nitro-*N'*-nitrosoguanidine, an agent known to cause transient cell arrest in the G1 or G2 phase of the cell cycle. PARP-1 modulates DNA repair via the base excision repair pathway to rejoin nicked strands of DNA. Overexpression of YY1 in HeLa cells stimulates catalysed PARP-1, resulting in accelerated DNA repair (Oei and Shi, 2001a, b). This, however, seems to act as a negative feedback; continuous overexpression of PARP-1 decreases YY1 affinity by poly-ADP-ribosylation at its DNA-binding sites and induces transcriptional silencing (Oei and Shi, 2001a, b). The system, therefore, functions to control gene modification and decrease production and overexpression of damaged genes. This process may have implications in the relief of genetic defects, senescence, and cancer.

Induction of YY1

Cellular localization and trafficking
Cellular localization of transcription factors to the nuclear matrix is essential for transcriptional regulation and control. McNeil *et al.* (1998) has identified specific sequences that lead YY1 to nuclear targets. Analysis of deletion constructs composed of Gal-4-tagged YY1 fusion proteins expressed in Hela cells and human Saos-2-osteosarcoma cells reveal the C-terminal domain (aa 256–341) as the chief constituent involved in high-affinity efficient targeting of YY1 to the nuclear matrix. The N-terminal domain of the protein permits a low-affinity association into the nucleus, but is not necessary, thus suggesting the significance of the C-terminus in nuclear localization as well as transcriptional repression.

Progression through the cell cycle also induces a DNA replication-associated switch in YY1 subcellular localization. As a DNA-binding protein, YY1 functions in the replication and regulation of the histone alpha complex, vital for proliferating cells (Palko *et al.*, 2004). YY1 nuclear localization and activity is significantly increased during the onset of the G1/S phase, followed by increased cytoplasmic localization in the late S phase with increased DNA-binding activity of YY1 and YY1-dependent histone genes (Palko *et al.*, 2004).

Molecular regulation of YY1
Indirect evidence.   Little is known regarding the regulation of YY1 activity. Several investigators have been able to demonstrate increased activity at promoters for genes such as histone deacetylase complex (HDAC) 1 and 2 in correlation with increased YY1 activity. Thus, indirect evidence exists to suggest that promoters, often in conjunction with cofactors such as msin3A, nuclear receptor corepressor (NCOR) and Sin3-associated polypeptide (SAP) 18/30, may regulate YY1 (Thomas and Seto, 1999). At the protein level, Hiromura *et al.* (2003) have shown in a murine retinoblastoma (Rb) model that alteration in YY1 chemical structure by *O*-linked *N*-acetylglucosaminylation frees YY1 to bind

DNA, resulting in transcriptional activation. These findings provide the basis for a mechanistic hypothesis published in 2001 demonstrating the net suppressive effects after inhibition of YY1 binding to HDAC promoter binding sites, also in an Rb model (Osborne *et al.*, 2001). Lastly, two models of post-translational cytoplasmic proteolytic activation are revealed in studies of the regulation of muscle development in primary skeletal muscle and cardiac cell lines. Loss of activation is achieved with proteolytic inhibition; these models are proposed to explain modulation of YY1 regulation in myoblast differentiation (Walowitz *et al.*, 1998). Human gene promoters that regulate YY1 are summarized in Table 2.

Direct evidence.   Direct activation by transcriptional activators has been shown only in a few models. Lee *et al.* (2004) suggests that bone morphogenic protein (BMP) induces GATA genes in an autocrine fashion and modulates YY1 transcriptional activity via the direct interaction of YY1 with BMP-activated SMADs. The transcription factor nuclear factor kappa B (NF-κB) has also been shown to regulate YY1. Elegant studies by Sepulveda *et al.* (2004) demonstrate concurrent direct binding of the Rel-B component of NF-κB to YY1 and sequences at the hs4 enhancer region of B-cell lymphoma *Igh* gene, thereby implicating this complex in the anti-apoptotic response and the upregulation of the proliferative potential of these lymphocytes *in vivo*.

YY1-mediated transcriptional regulation

Mechanisms of YY1-mediated transcriptional repression
It has been suggested that YY1 represses transcription using multiple mechanisms. Most frequently, these mechanisms involve the competition of YY1 with activating factors in overlapping binding sites, thereby decreasing promoter activity and resulting in transcriptional repression. Other hypotheses include the negative regulation of YY1 on neighboring promoter-bound activators (Shi *et al.*, 1997). As delineated by both Shi *et al.* (1997) and Thomas and Seto (1999), there are three models that explain YY1 as a transcriptional activator.

The displacement model (Figure 4).   Accumulating evidence suggests the presence of many promoters with sequences of YY1 sites that overlap and compete with activating factors, including serum response element (SRE) of the cellular FBJ/FBR osteosarcoma *(s-fos)* gene, α-actin muscle regulatory elements (MREs), and the muscle creatine kinase CarG motif (Shi *et al.*, 1997) have demonstrated overlapping sites that compete with YY1 for occupancy. YY1 competition with MREs suggests its significance in modulating myoblast maturation and differentiation. Likewise, competition of YY1 with the β-casein activating promoter of mammary epithelial cells, known as mammary gland factor (MGF), results in transcriptional repression. Transcriptional

Table 2 Human gene promoters that regulate YY1

Promoter/gene product	Activity on YY1	Interacting factors	References
HDAC[a]-dependent HD1 (histone deacetylase-human homologue of the yeast RPD3 gene)	*Repression* Repression via binding to YY1 glycine-rich domain		Shi *et al.* (1997)
HDAC-1,-2 complexes	Repression via complex association; interaction with Mad and nuclear hormone receptors (mSin-3A, N-CoR); chromatin remodeling (CHD-3,-4, SAP30); association with chromatin assembly factor 1 (RbAp48); and binding methylated DNA sequences (MeCP2)	mSin3A, N-CoR, SAP18/30, RbAp46/48, CHD3/CHD4, MeCP2, SAP30	Thomas and Seto (1999), Huang *et al.* (2003) and Nguyen *et al.* (2004)
HDAC-3	Independent repression complex		Thomas and Seto (1999), Huang *et al.* (2003) and Nguyen *et al.* (2004)
HDAC-independent GATA-1	*Repression* Corepression of ε-globin gene		Shi *et al.* (1997)
Nucleophosmin (Nuclear phosphoprotein)	Repression		Brankin *et al.* (1998)
PARP-1	Repression and transcriptional silencing during increased DNA damage and catalysed PARP-1 expression		Oei and Shi (2001b)
Smad-1, -2, -4	Corepression of TGF-β-induced epithelial to mesenchyme transition		Morgan *et al.* (2004)
HAT[b]-dependent CBP	*Activation* Required to activate CREB-dependent promoters through histone modification		Thomas and Seto (1999) and Huang *et al.* (2003)
P300	Required to activate E1A oncoprotein through histone modification		Shi *et al.* (1997), Thomas and Seto (1999) and Huang *et al.* (2003)
HAT-independent B23 nucleolar protein	*Activation* Relief of repression		Shi *et al.* (1997)
BrdU	Activation and enhancement of YY1 expression	SRF	Shi *et al.* (1997) and Lee *et al.* (1992)
C/EBP β protein	Coactivation of the HPV-18 promoter		Shi *et al.* (1997)
E1A oncoprotein	*Trans*-activation (conversion from repressor to activator)	P300	Shi *et al.* (1997), Thomas and Seto (1999) and Huang *et al.* (2003)
MGF (mammary gland specific factor)	Relief of repression (only during lactation) via competition/displacement		Shi *et al.* (1997)
NF-κB	Relief of repression		Lu *et al.* (1994) and Shi *et al.* (1997)
TBP (TATA-binding protein); TAF (TBP-associated factor)	Coactivation via interaction with acidic activation domains	Sp1, USF, CTF, E1A oncoprotein	Chiang and Roeder (1995), Thomas and Seto (1999) and Nguyen *et al.* (2004)
YY1AP (HCCA2)	Coactivation		Wang *et al.* (2004)

repression is reversed by the alternative competition/displacement model. In the MGF model, when lactation results in increased MGF concentration, YY1 is displaced from its overlapping site on the β-casein promoter, resulting in baseline activation (Shi *et al.*, 1997). Expression of other transcription factors such as NF-κB may also increase, thereby displacing YY1 and relieving repression, such as that demonstrated in serum amyloid gene transcription in hepatoma cell lines (Lu *et al.*, 1994).

Interference with the function of transcriptional activators (Figure 5). It has been established that the c-fos promoter not only contains overlapping sites for YY1 and SRE, but also possesses two additional YY1 sites between the calcium/cyclic AMP response element (CRE) and the TATA box. YY1 adheres distally, resulting in repression of the upstream CRE promoter (Figure 5a, Direct inhibition). YY1 can repress the c-fos promoter in either a binding site-dependent or binding site-independent manner (Shi *et al.*, 1997), both of
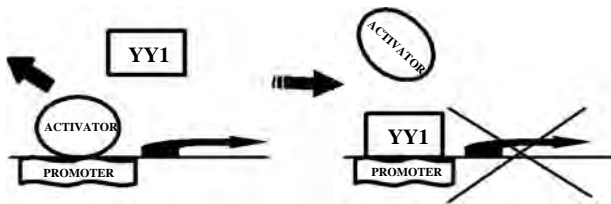
Figure 4 Activator-displacement-induced repression model of YY1-mediated transcriptional repression. YY1 can effect transcriptional repression at promoter binding sites following competition with activators with subsequent activator displacement. This has been demonstrated in the myoblast YY1/α-actin MRE interaction and the YY1/muscle creatine kinase CarG motif interaction. Both genes are known to possess overlapping binding sites that compete with YY1 for occupancy.
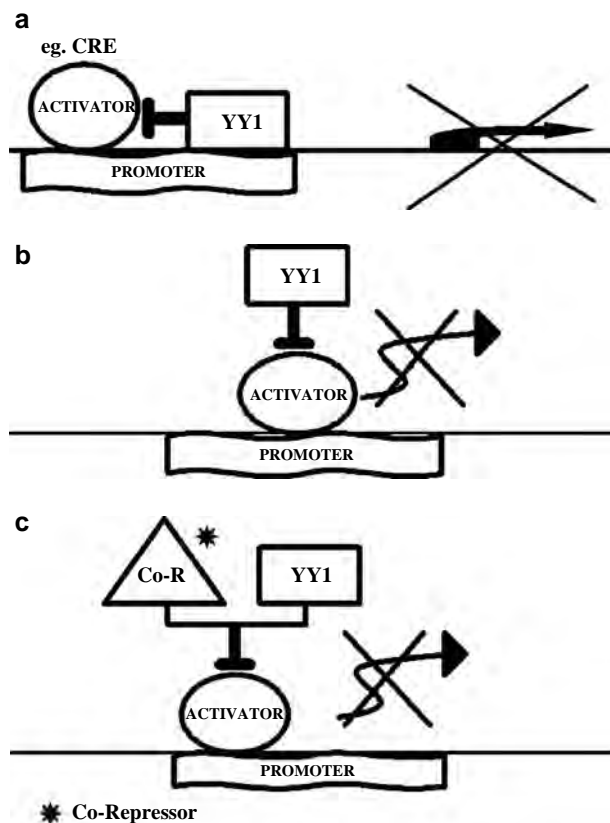

Figure 5 Models of YY1-mediated inhibition of transcriptional activation. (**a**) YY1 directly represses transcriptional activation. Despite the presence of a bound transcriptional activator to a gene promoter site, YY1 may adhere distally, resulting in repression of the upstream promoter. Such is the case with the c-*fos* promoter, possessing two additional YY1 sites between the CRE and the TATA box. (**b**) YY1 exerts transcriptional activator inhibition via direct physical binding. YY1 interferes with the action of a transcriptional activator, thus causing transcriptional repression. An example of binding-site-dependent interference involves the c-*fos* promoter, stimulated to transcriptional activation by binding at the CREB site, but transformed to repression due to the interaction of the YY1 zinc-finger motifs and the basic leucine zipper region (bZIP) on the CREB protein. (**c**) YY1 exerts transcriptional activation: inhibition by interaction. The DNA-bending model, suggesting the ability of YY1 to interfere with the communication of CREB and consequently retard CREB-mediated activation, represents an example of a binding site-independent reaction independent of physical interactions with DNA and gene promoter sites.

which involve the interaction of the zinc-finger motifs on YY1 and the basic leucine zipper region (bZIP) on the cAMP response element binding (CREB) protein. The presumed manner by which YY1 and CREB interact in the nucleus (Guo *et al.*, 1997) and lead to the transcriptional repression of CREB represents an example of a binding site-independent reaction (Figure 5b). Galvin and Shi (1997) argue against the DNA-binding model, demonstrating the ability of YY1 to interfere with the communication of CREB and consequently retard CREB-mediated activation (Figure 5b) independently of physical interactions with DNA. As a potent coactivator of YY1, E1A can block YY1-induced repression by disturbing the YY1–CREB interaction (Chang *et al.*, 1989; Shi *et al.*, 1991, 1997; Yao *et al.*, 1998).

Interactions with corepressors (Figure 6). A third model exemplifies the ability of YY1 to recruit corepressors that directly act to facilitate transcriptional repression or induce chromatin remodeling/condensing to further assist YY1-mediated DNA interaction and repression (Thomas and Seto, 1999). The zinc-finger and glycine-rich regions of YY1 are known to be instrumental in YY1 repression activity. Simultaneous deletions in both regions and/or each individual region render GAL4-YY1 fusion proteins insufficient for transcriptional repression. Additionally, YY1 often requires the help of cofactors that interact with its repression domains to facilitate repression. Such cofactors include mRPD3, a mouse homologue of RPD3 protein which was shown to enhance transcriptional repression when overexpressed in GAL4-YY1 fusion proteins; GATA-1, involved in the corepression of the ε-globin gene (Yang *et al.*, 1996); and Smad family members (Kurisaki *et al.*, 2003), involved in the inhibition of TGF-β-induced epithelial to mesenchyme transition.

YY1 is also capable of repressing cofactors pivotal to cellular activity and viral regulation, including interferon beta (IFN-β) and gamma (IFN-γ). YY1 binding to the IFN-β promoter may activate or repress transcriptional activation of IFN-β depending on its association with HDACs (Weill *et al.*, 2003). It also associates with nuclear factor AP2 to form protein complexes that relieve transcriptional activation of IFN-γ (Ye *et al.*,
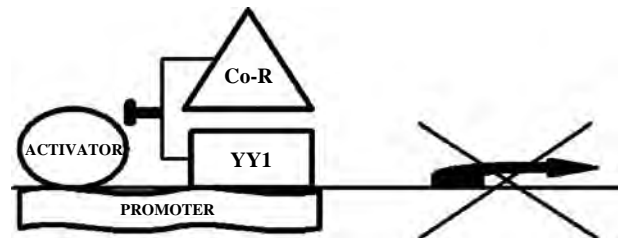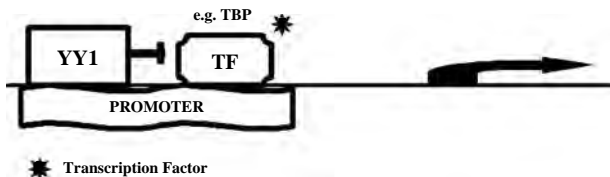

Figure 6 YY1-mediated repression of activators via corepressor complexes. YY1 mediates transcriptional activator repression by complexing with corepressors. YY1 may recruit corepressors that directly act to facilitate transcriptional repression or induce chromatin remodeling/condensing to further assist YY1-mediated DNA interaction and repression.

1994). Two mechanisms that may therefore account for YY1-mediated transcriptional regulation of gene products such as interferons include (1) a similar displacement competition with YY1 and an AP-1 overlapping site and (2) YY1-mediated repression only in the presence of a neighboring site binding an AP-2-like protein (Shi *et al.*, 1997).

Direct activation (Figure 7). The first model proposes direct interaction of YY1 with transcription factors that stimulate YY1-mediated transcriptional activation, such as TATA-binding protein (TBP), (TBP)-associated factors (TAFs), and transcription factor IIB (TFIIB) (Nguyen *et al.*, 2004). It is likely that YY1 uses two acidic activation domains to accomplish this. However, studies have shown interactions with cofactors that act distal to the N-terminus, suggesting a possibility of a complex regulation of repression activity exceeding activation (Thomas and Seto, 1999). This model may therefore represent an oversimplified model for YY1-mediated activation.
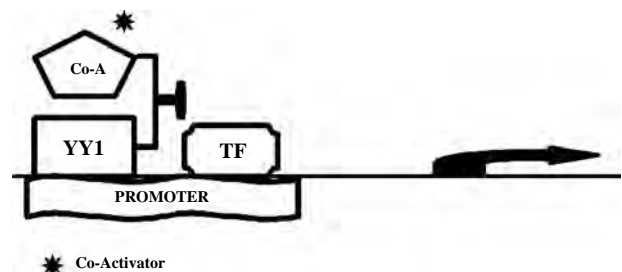
Cofactor-induced inhibition of YY1 repression (Figure 8). The second model proposes a mechanism that induces the masking and unmasking of repression (C-terminus) and activation (N-terminus) domains, respectively. It is possible that YY1 interacts with other cellular factors to unmask the N-terminal activation domain, perhaps by undergoing structural alterations including changes in the C-terminus, thereby inhibiting the constitutive YY1 repression (Thomas and Seto, 1999). In addition, the C-terminus may play an important role in masking the N-terminal activation region; studies in which the YY1 C-terminal domain was deleted resulted in the exposure of N-terminal sequences and a significant enhancement in the transcriptional activation of YY1 (Thomas and Seto, 1999).

Recruitment of coactivators (Figure 9). Thirdly, YY1 may act as an indirect activator of transcription by recruiting other transcription activating factors. It primarily induces cofactors to tether directly to the target promoter and initiate activation (Thomas and Seto, 1999). As was seen in the repression models, YY1 has also been found to interact with coactivators with histone acetyltransferase (HAT) activity, such as CBP and p300 (Lee *et al.*, 1998). YY1 may recruit p300, thereby facilitating chromatin expansion to provide better DNA interactions. This mechanism allows for an easier manner by which YY1 can carry out transcriptional activation (Lee *et al.*, 1995). These findings provide a strong consensus that supports the direct interaction of cofactors for YY1-induced activation.
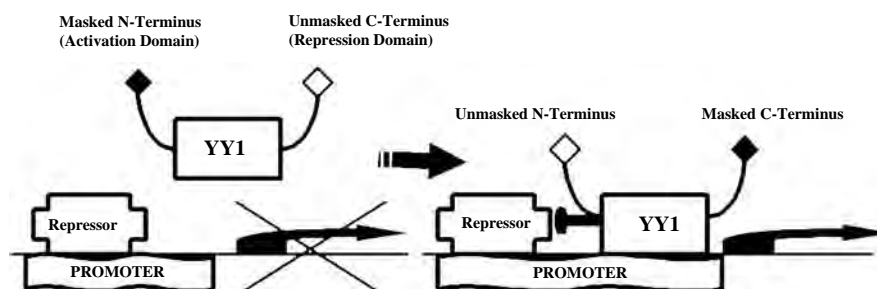

Figure 7 Direct activation by YY1. YY1 may directly activate gene transcription by binding to gene promoters and/or transcription factors that stimulate YY1-mediated transcriptional activation. Known examples include TATA-binding protein, TBP-associated factor II55, and transcription factor IIB.

Figure 9 YY1-mediated activation via recruitment of coactivators (Co-A). YY1 may act as an indirect activator of transcription by inducing cofactors to tether directly to the target promoter and initiate activation. Coactivators with histone acetyltransferase (HAT) activity such as CBP and p300 are likely candidates. YY1 may also recruit p300, thereby facilitating chromatin expansion to provide better DNA interactions.

Figure 8 Indirect activation by YY1. Activation via cofactor-induced inhibition of YY1 repression. The net result of separate or combined induction of C-terminus (repression) domain masking or N-terminus domain (activation) unmasking of repression is transcriptional activation. It is possible that YY1 may interact with cellular factors to undergo structural alterations, thereby inhibiting constitutive YY1 repression. The C-terminus domain itself may play an important role in masking the N-terminal activation region in an autocrine fashion. Studies in which the YY1 C-terminal domain was deleted resulted in the exposure of N-terminal sequences and a significant enhancement in YY1-mediated transcriptional activation.

Cofactors involved in YY1-mediated transcriptional regulation

Coactivators of YY1

YY1 may act independently. However, as noted previously, many of the effects of YY1 on gene transcription are executed via cofactors (Table 2). The AAV protein E1A is an example of a coactivator of YY1. Possible mechanisms involved in such collaborative activation might include protein–protein interactions with E1A/p300 complexes required for E1A to relieve YY1-mediated repression (Wang *et al.*, 1993), and DNA binding (Kim and Shapiro, 1996; Shi *et al.*, 1997). In addition, Wang *et al.* (1993) and Shi *et al.* (1991) independently demonstrated that E1A may serve as an initiator of YY1-mediated transcriptional activation via attachment to the P5 promoter. More recent studies screening for cellular proteins in the HeLA cDNA library have identified a novel protein, YY1-associated protein (YY1AP), a ubiquitous protein expressed in normal human tissue and metastatic cell lines. YY1AP was shown to colocalize into the nuclear matrix with YY1 and enhance its transcriptional activation *in vivo* and *in vitro* (Wang *et al.*, 2004).

Corepressors of YY1

Yang *et al.* (1996) was able to isolate and identify mRPD3 by yeast two-hybrid assay revealing an identical glycine-rich domain (a necessary component for transcriptional repression) to that of YY1. Overexpression of mRPD3 significantly increased the ability of Gal4-YY1 fusion proteins to repress transcription, suggesting a possible role of mRPD3 as a corepressor of YY1.

SAP30 is also required for the normal functioning of RPD3, an alternate corepressor of YY1. SAP30 alone, however, is not sufficient for transcriptional repression, and is thus dependent on transcription factors such as YY1 to tether it to the promoter (Huang *et al.*, 2003). The *in vivo* presence of this complex suggests one of many different YY1-dependent mechanisms of transcriptional repression.

The function of YY1 in transcription is context-specific and requires interactions with many cellular factors. As a result, YY1 develops intracellular networks that allow it to induce multiple functions in transcriptional initiation, activation and repression, ultimately leading to the regulation of normal cell growth and survival. As previously noted, it is apparent that YY1 expression and localization can be coordinated with phases of the cell cycle (Palko *et al.*, 2004); it is via the study of the putative interactions between YY1 and cell cycle regulators, death genes, and transcription factors and cofactors that mechanistic evidence has surfaced to support the role of this ubiquitous transcription factor in the suppression or progression of various malignancies. The human promoters/gene products regulated by YY1 are summarized in Table 3.

YY1 and cancer biology

In order to determine the potential impact of YY1 activity on tumorigenesis, a brief review of key cell cycle regulators, patterns of cell cycle dysregulation resulting in cancer, and known interactions between these regulators and YY1 is warranted. The two cell division events that control progression to replication are (1) entry into the S phase during which time DNA is replicated (G1 (first gap)/S (DNA synthesis) checkpoint), and (2) entry into the M-phase when mitosis occurs (G2 (second gap)/M checkpoint). CDK4/6-cyclin D and CDK2-cyclin E and the transcription complex that includes retinoblastoma (Rb) and E2F control the G1/S checkpoint. Phosphorylation of Rb by CDK 4/6-cyclin D and CDK2 dissociates the Rb-repressor complex, permitting rapid and transient transcription of S-phase-promoting genes (Bartek and Lukas, 2001). Expression of the proto-oncogene c-*myc* can influence cyclin D activity at this checkpoint (Amati *et al.*, 1998). In addition, G1/S progression is associated with maximal phosphorylation of the ubiquitous DNA-associated protein DEK, which is thought to function as a transcription factor modulator (Kappes *et al.*, 2004). Alternatively, the tumor suppressor transcription factor p53 may be activated at the G1/S checkpoint in response to DNA damage. Activation of p53 results in CDK**2** inhibition, allowing for delay in the progression of the cell cycle for purposes of repair via poly (ADP-ribose) polymerase-1 (PARP-1), p21, and/or the negative regulator murine double minute 2 (Mdm2), an oncoprotein whose amplification and/or overexpression occurs in a wide variety of human cancers. Once repair is complete, progression to S phase proceeds. After S phase transcriptional activity is complete, dephosphorylation of a tyrosine residue within cell division control 2 (CDC2), the catalytic subunit of the cyclin/CDK heterodimer, signals activation late in G2 to activate G2/M progression (Zhao and Elder, 2005). HDAC's are critical at this juncture; they may enhance or inhibit progression at the G2/M checkpoint via DNA binding (Figure 10).

Therefore, uncontrolled cell cycle progression is a major event in tumorigenesis. Multiple mechanisms should act in concert in order to prevent uncontrolled cell division. Some of these are intrinsic molecules in the cell that regulate the transition from and between different phases of the cell cycle (i.e., CDKs, cyclins, pRB, p53, MDM$_2$, c-Myc, etc.), whereas others are signaling mechanisms sensing the environment that prompt a cell to remain in homeostatic balance with its surrounding tissue.

Regulation of the cell cycle by YY1

The putative role of YY1 in tumorigenesis is supported by its known interaction with the cell cycle regulation.

YY1 and cyclin D. The association of YY1 with cell cycle signaling pathways has been reported by Cicatiello *et al.* (2004), who noted that cyclin D1 gene promoter

**Table 3** Human promoters/gene products regulated by YY1

Promoter/gene product	Effect of YY1	Interacting factors	References
Repression			
α-actin	Repression by IL-1β pathway (controlled by YY1)	Norepinephrine in cardiomyocites, SRE, TATA	Shi *et al.* (1997), Thomas and Seto (1999), Kurisaki *et al.* (2003) and Wang *et al.* (2004)
β-casein	Repression via displacement/competition with MGF	MGF (STAT family member)	Shi *et al.* (1997), Thomas and Seto (1999) and Raught *et al.* (1994)
ε-globin gene	Repression	GATA-1	Shi *et al.* (1997) and Thomas and Seto (1999)
CAT[a]	Repression in the absence of E1A oncoprotein	TATA	Shi *et al.* (1997) and Thomas and Seto (1999)
CREB, Sp1	Binding site-independent repression by interference with communication between the promoters and their transcription machinery		Guo *et al.* (1997), Galvin and Shi (1997), Shi *et al.* (1997) and Huang *et al.* (2003)
CXCR4 (chemokine receptor)	Repression		Nguyen *et al.* (2004)
GCM-CSF	Repression	AP-1, Sp1-related binding	Shi *et al.* (1997)
IFN-β	Repression	HDAC[b] complexes	Weill *et al.* (2003)
IFN-γ	Repression via displacement/competition or activation	AP-1,-2, Sp1, NFAT	Ye *et al.* (1994), Shi *et al.* (1997) and Sweetser *et al.* (1998)
Immunoglobin κ 3′ enhancer	Repression		Shi *et al.* (1997), Thomas and Seto (1999) and Wang *et al.* (2004)
Involucrin	Repression	AP-1, TATA	Shi *et al.* (1997)
MLP[c]/Ad12	Repression	TATA	Shi *et al.* (1997)
Muscle creatine kinase CArG motif	Repression via displacement/competition		Shi *et al.* (1997)
Muscle regulatory element (MRE)	Repression via displacement/competition		Shi *et al.* (1997)
PAI-1[d] and Id-1[e] of Smad superfamily	Repression of promoters for TGF-β- and BMP[f]-specific transcription and cell differentiation	Mad domain of Smads 1-4, SBE	Kurisaki *et al.* (2003) and Morgan *et al.* (2004)
Serum response element (SRE) of c-fos	Repression via displacement/competition	CRE, SRF, TATA	Shi *et al.* (1997), Thomas and Seto (1999), Nguyen *et al.* (2004), Wang *et al.* (2004) and Lee *et al.* (2004)
Viral promoters			
P5 promoter of adeno-associated virus (AAV)	Repression of AAV in absence of E1A; displacement/competition	TATA, MLTE, YY1+1	Shi *et al.* (1997), Thomas and Seto (1999), Huang *et al.* (2003) and Wang *et al.* (2004)
Major IE enhancer- promoter of cytomegalovirus	Repression	TATA, NF1, NF-κB	Shi *et al.* (1997)
BZLF1-viral gene product of Epstein–Barr Virus	Repression of BZLF1 (maintains virus in latent state)	TRE, AP-1-like, ZEBRA	Shi *et al.* (1997) and Thomas and Seto (1999)
ALK5/6-active receptors of green fluorescent protein adenovirus	Repression of ALK-mediated preosteoblastic differentiation	TGF-β, BMP	Kurisaki *et al.* (2003)
ALP-early differentiation marker of green fluorescent protein adenovirus	Repression of ALP-mediated cell differentiation	Smad-1, -4	Kurisaki *et al.* (2003)
Long terminal repeat (LTR) of human immunodeficiency virus type 1	Repression (maintains viral latency)	Tat, USF, LBP-1	Shi *et al.* (1997)

Table 3 (*continued*)

Promoter/gene product	Effect of YY1	Interacting factors	References
E6 and E7-oncoproteins of human papillomavirus type 16	Repression of HPV-specific long control region (LCR)	AP-1, CAAT, Sp1, E2	Shi *et al.* (1997)
Upstream regulatory region of human papillomavirus type 18	Repression when switch region off (no C/EBP β protein bound)	TATA, AP-1, C/EBP β	Shi *et al.* (1997) and Thomas and Seto (1999)
Activation B-type natriuretic peptide gene	Activation		Wang *et al.* (2004)
CREB	Activation	E1A (reversal of YY1-mediated repression of CREB)	Guo *et al.* (1997), Galvin and Shi (1997), Shi *et al.* (1997) and Huang *et al.* (2003)
C-Myc	Activation	TATA, NF-κB, PRF, Sp1	Shi *et al.* (1997), Thomas and Seto (1999), Wang *et al.* (2004) and Nguyen *et al.* (2004)
Col1a1	Activation		Wang *et al.* (2004)
Dr-α	Activation		Shi *et al.* (1997)
Gp91	Activation		Wang *et al.* (2004)
GRP78	Activation (only under stress-induced conditions)	dbpA, YB-1, CBF/NF-Y	Shi *et al.* (1997) and Thomas and Seto (1999)
Histone H4 gene	Activation by anchoring promoter to nuclear matrix (chromatin remodeling)		Shi *et al.* (1997)
IL-1β pathway (repression of α-actin)	Activation	Norepinephrine, SRE, TATA	Shi *et al.* (1997), Thomas and Seto (1999), Kurisaki *et al.* (2003) and Wang *et al.* (2004)
LINE-1 (human transposable element)	Activation	RNA polymerase III	Shi *et al.* (1997)
Msx2	Activation		Wang *et al.* (2004)
Myelin pyroxidal phosphate	Activation		Wang *et al.* (2004)
p53	Activation or inhibition	NF1, C/EBP, PF1, HSF, NF-κB, bHLH, p300	Shi *et al.* (1997), Thomas and Seto (1999), Wang *et al.* (2004) and Nayak and Das (2002)
PARP-1	Activation and subsequent increase in DNA repair		Oei and Shi (2001b)
Viral promoters P6/B19 parvovirus	Activation	TATA	Shi *et al.* (1997) and Thomas and Seto (1999)
URR/HPV-18	Activation when switch region on (C/EBP β protein bound)	TATA, AP-1, C/EBP β	Shi *et al.* (1997) and Thomas and Seto (1999)
VP5/HSV-1 (promoter region of herpesvirus type 1)	Cofactor-independent activation of long terminal repeat	NF1, Sp1, TATA	Shi *et al.* (1997) and Thomas and Seto (1999)
Initiation P5/AAV	Initiation of transcription by binding P5 promoter	TATA, MLTE, YY1+1	Shi *et al.* (1997), Thomas and Seto (1999), Huang *et al.* (2003) and Wang *et al.* (2004)
PCNA	Initiation	ATF, RFX1	Shi *et al.* (1997)

activation in estrogen-responsive human breast cancer is marked by release of the YY1 transcriptional repressor complex including HDAC 1 and is sufficient to induce the assembly of the basal transcription machinery on the promoter and to lead to initial cyclin D1 accumulation in the cell. Upon estrogen stimulation, the cyclin
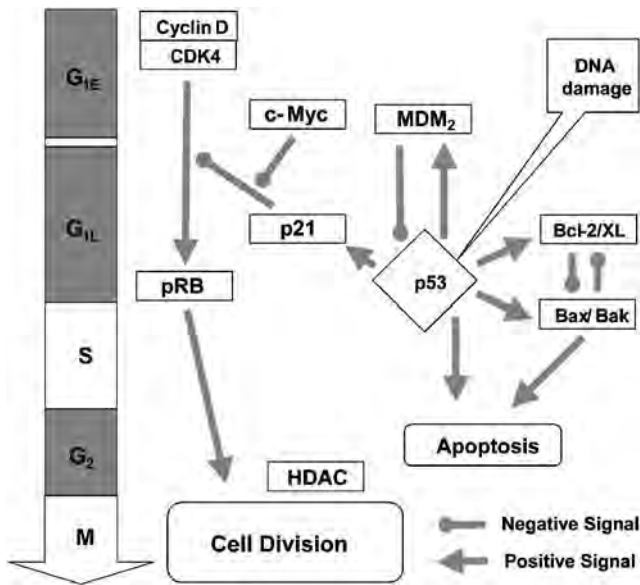
Figure 10 Cell cycle, tumorigenesis and YY1. The cell cycle is a coordinated sets of events resulting in cell growth and cell division or proliferation. It can be described by four phases composed of M phase (mitosis), G_1 phase (gap or growth 1), S phase (DNA synthesis) and G_2 phase (gap or growth 2). Multiple mechanisms should act in concert in order to prevent uncontrolled cell division. Some of these are intrinsic molecules in the cell that regulate the transition from and between different phases of the cell cycle (i.e., CDKs, cyclins, pRB, p53, MDM_2, c-Myc, etc.). Uncontrolled cell cycle progression is a major event in tumorigenesis. There is a dynamic interaction between the activity of the transcription factor YY1 and different components of the cell cycle and its check points. These interactions frequently result in a dysfunctional cell cycle progression and possibly tumorigenesis.

D1/CDK4 holoenzyme associates with the cyclin D1 promoter, where E2F and pRb can also be found, contribute to the long-lasting gene enhancement required to drive G1-phase completion.

YY1 and p53. Extensive evidence supports the role of YY1 in tumorigenesis via its association with the tumor suppressor gene product p53. Both expression and function of p53 are tightly regulated by post-translational modifications such as phosphorylation, ubiquitination, and acetylation with the goal of preservation of genomic integrity. In transfection experiments, YY1 inhibits p53-activated transcription from the p53-binding site that contains the ACAT sequence. Furthermore, YY1 and p53 are noted to be colocalized around the nucleoli and in discrete nuclear domains in an *in vitro* model of apoptosis in PC-12 rat adrenal tumor cells (Yakovleva *et al.*, 2004). YY1 may attenuate p53-dependent transcription from a subset of p53 target genes, a hypothesis that may be relevant for defining the role of YY1 in directing cells either to growth arrest or apoptosis upon p53 binding. Further in-depth models, described below, are offered by several investigators:

YY1 disrupts p53/p300 interaction. Sui *et al.* (2004) demonstrated that YY1 interacts with p53 and inhibits its transcriptional activity by disrupting the interaction between p53 and its coactivator p300, thereby blocking p300-dependent p53 acetylation and stabilization, and disabling this checkpoint mechanism. Ablation of endogenous YY1 results in p53 accumulation due to a reduction in p53 ubiquitination and increased expression of p53 target genes in response to genotoxic stress *in vivo*. Conversely, YY1 overexpression stimulates p53 ubiquitination and degradation, thereby supporting the hypothesis that increased YY1 expression and activity inhibits the accumulation of p53.

p53 enhances murine double minute 2-mediated p53 inactivation. Alternatively, YY1 is known to interact with the negative regulator murine double minute 2 (mdm2), an oncoprotein whose amplification and/or overexpression occurs in a wide variety of human cancers. YY1 promotes the assembly of the p53–mdm2 complex, enhancing the mdm2-mediated ubiquitination and subsequent inactivation of p53 (Gronroos *et al.*, 2004). Likewise, studies demonstrating the direct physical interaction between Hdm2 (the human homologue of Mdm2) and p53 show that the basis for YY1 regulation of p53 ubiquitination is its ability to facilitate Hdm2/p53 interaction (Sui *et al.*, 2004). Significantly, recombinant YY1 is sufficient to induce Hdm2-mediated p53 polyubiquitination *in vitro*, suggesting that this function of YY1 is independent of its transcriptional activity. These findings identify YY1 as a potential cofactor for Mdm2 and Hdm2 in the regulation of p53 homeostasis and indicate a possible role for YY1 in tumorigenesis.

YY1 binds the neurofibromatosis promoter site. YY1 has been shown to bind p53 at the neurofibromatosis 1 (NF1)/YY1 promoter binding site (Nayak and Das, 1999). NF1 is the mutant gene mapped to chromosome 17 and known to cause the tumor von Recklinghausen neurofibromatosis. Interestingly, Nayak and Das (2002) have proven this binding to be absent in tumor cells expressing the proapoptotic gene Bax. Taken together or independently, the above models provide molecular clues as to the mechanism of YY1-mediated modulation of p53 with the potential for consequent regulation of resistance to apoptotic stimuli.

YY1 and c-myc. The proto-oncogene c-*myc* possesses a key role in cellular processes such as proliferation, differentiation, apoptosis, and transformation (Riggs *et al.*, 1993; Shrivastava *et al.*, 1996). C-*myc* activity has been implicated in the pathogenesis of malignances such as breast, ovarian, prostate, hepatocellular, and colorectal carcinoma as well as lymphoma and plasma cell tumors. Riggs *et al.* (1993) and Shrivastava *et al.* (1996) independently demonstrated in murine and human tumor models that YY1 can activate both endogenous and exogenous c-*myc* promoters when overexpressed. In turn c-myc overexpression appears to alter the constitutive repressive role of YY1 by interfering with the association between YY1 and basal

transcription proteins such as TATA-binding protein and transcription factor IIF, with altered transcription of target genes (Austen *et al.*, 1998).

Translational evidence of the significance of these findings has been demonstrated in a murine model of hepatocellular carcinoma (HCC) whereby YY1 binding is blocked in a model of *N*-nitrosodiethylamine-induced hepatocarcinogenesis, with concomitant c-myc over-expression. Reversal of tumor formation with dodecanol-limonene, a monoterpene monocyclic compound with an unknown mechanism of cancer chemoprevention, is associated with a constitutive (high) level of YY1 binding along with inhibition of c-myc over-expression as seen in non-tumorous liver tissue. Hence, YY1 may constitutively serve to repress c-myc responsive antiapoptotic signals (Parija and Das, 2003).

YY1 and retinoblastoma Rb. It is known that YY1 binds with the retinoblastoma (Rb) protein to accelerate cellular progression to S phase, thereby potentiating cellular proliferation and tumorigenesis. Petkova *et al.* (2001) postulate that the responsible mechanism is the Rb-YY1 heterodimerization resulting in inhibition of the transcription factor due to binding destabilization. Conversely, both inhibition of the of YY1–Rb complex as well as inhibition of HDAC binding at the promoter reverses YY1 transcriptional activation, potentially altering acceleration of cell cycle mechanics with resultant loss of malignant potential (Osborne *et al.*, 2001; Hiromura *et al.*, 2003).

YY1 and HDACs. YY1 binds replication-dependent histone genes to effect proliferation and chromatin remodeling for accelerated replication via HDACs (Guo *et al.*, 1997; Thomas and Seto, 1999; He and Margolis, 2002; Huang *et al.*, 2003). The state of HDAC activation is dependent upon acetylation (active) versus deactylation (inactive). Studies by Palko *et al.* (2004) reveal changes in YY1 subcellular localization in CHO and HeLa cells, specifically, upregulation of the histone gene family at the G1/S boundary and subsequent down-regulation at the mid-point of the S phase to correlate with YY1 activity. These data suggest an intimate association between YY1 activity and HDAC activation. Thus, YY1 localization appears to be coupled to DNA synthesis and responsive to cell cycle signaling pathways, lending credence to the hypothesis that proliferation and furthermore, resistance to apoptosis may be mediated via YY1 regulation of HDACs.

YY1 regulation of cell death
Cell proliferation and cell death are two functionally opposing cellular fates that paradoxically share many, nonoverlapping, molecular interdependent components and regulatory signals. The two processes are coupled at various levels through the individual molecular player responsible for orchestrating cell expansion. Moreover, the same molecular components are targets for oncogenic changes that frequently drive cell proliferation to cooperate with those that uncouple proliferation from

apoptosis during transformation and tumorigenesis (Evan and Vousden, 2001; Fridman and Lowe, 2003). Consequently, alteration in either or both processes, uncontrolled cell proliferation and impaired cell death, might synergize toward tumorigenesis.

As we have discussed above, YY1 interacts with many elements involved in cell cycle with an overall outcome of regulation of positive signals promoting cell proliferation (i.e., p53, MDM2, cyclin D, etc.). In addition, YY1 has been implicated in the regulation of the activity and expression of apoptosis-related molecules (i.e., NF-κB, Fas, DR5, etc.). It would not be surprising that deregulated YY1 activity might serve as central molecule causing dysfunctional cell proliferation and increased resistance to cell death, therefore promoting tumorigenesis (Figure 10).

Apoptosis. Apoptosis is a cellular suicide program that eradicates excess or potentially dangerous cells. Its important physiological functions include terminating immune responses and eliminating infected or cancerous cells. The induction of apoptosis relies critically on the activation of caspases, a family of proteinases that kill the cell via proteolysis of key substrates. Two main pathways have been defined that initiate caspase activation. The first begins at the cell surface and involves ligand-induced activation of death receptors (e.g., Fas, TNF-R1, DR4, DR5), which then recruit and activate caspases. The second involves mitochondrial integration of cellular stress signals and mitochondrial dysregulation with release into the cytosol of cyto-chrome *c*, which activates caspase via the adaptor molecule, apoptotic protein factor 1 (APAF-1). In certain cells, the induction of apoptosis by death receptor signaling is complemented by mitochondrial activation. The apoptotic pathways are regulated at multiple levels such as by inhibitors of apoptosis family (IAPs) and the Bcl-2 family. Defects in the regulation of apoptosis contribute significantly into the pathogenesis and progression of most cancers. Apoptotic defects also contribute to tumor cell resistance to chemotherapy, radiotherapy, hormonal therapy, and immune-based treatments. Alterations in the expression and function of several apoptosis regulatory genes have been demonstrated in many cancers suggesting new targets for drug discovery (Wolf and Green, 2002; Reed, 2004). A few examples will be provided below in which YY1 is shown to play an important role in the regulation of apoptotic signaling pathways.

YY1 and NF-κB. The observation that the NF-κB family of transcription factors plays a key role in the regulation of immune and inflammatory responses as well as apoptosis has led several investigators to study the role of this factor and its regulated genes in the process of tumor progression and metastasis. Mounting evidence from several models, including metastatic murine colon cancer (Luo *et al.*, 2004), B-cell lymphoma (Sepulveda *et al.*, 2004; Jazirehi *et al.*, 2005), murine breast cancer (Rahman and Sarkar, 2005), and murine

cholangiocarcinoma (Chen *et al.*, 2005) suggest that NF-κB may modulate the apoptotic response. Furthermore, Huber *et al.* (2004) noted that NF-κB is necessary for epithelial to mesenchymal transition, a process that facilitates tumor metastasis. Mechanisms for NF-κB regulation in tumorigenesis, however, remain unclear. It is now known that YY1 and NF-κB may interact in multiple ways: Sepulveda *et al.* (2004) describe binding of the Rel-B NF-κB subunit to YY1 with the subsequent complex binding an enhancer region of the genome of a B-cell lymphoma line to increase IgH chain expression. Chan *et al.* (1996) have identified a serum response factor/NF-κB-like element containing potential overlapping core recognition binding motifs for YY1 in the cytomegalovirus promoter, a virus associated with immunosuppression-related lymphoma. Lastly, a cytokine response unit within the serum amyloid A gene promoter that binds NF-κB contains an overlapping binding motif for YY1. YY1 binding at that site was shown to effectively inhibit NF-κB binding and transcriptional activity (Lu *et al.*, 1994). Thus, indirect evidence exists that would suggest that NF-κB and YY1, when simultaneously transcriptionally active, may act in concert to exert synergistic or opposing effects, depending upon the cellular context and stimulus for gene regulation.

YY1 and proinflammatory cytokines. Reports of tumor and peritumoral proinflammatory cytokine production have been a recurring theme in the oncologic literature. Gene products such as inducible nitric oxide synthese (iNOS), tumor necrosis factor alpha (TNF-α), interleukin–1 (IL-1), and IFN-γ are elaborated in an autocrine and/or paracrine fashion in several cancer models and their expression has been shown to modulate response to apoptotic stimuli. Specifically, TNF-α is known to engage the TNF receptor and mediate apoptosis (Laster *et al.*, 1988); NO has been shown in several systems to induce apoptosis both via mitochondrial and Fas death pathways as well as by p53 and NF-κB modulation (Li *et al.*, 2004; Sagoo *et al.*, 2004); IL-1 appears to play a critical role in models of lipopolysaccharide-induced apoptosis (Hilbi *et al.*, 1997), and IFN-γ can also induce p53-independent apoptosis (Kano *et al.*, 1997; Ossina *et al.*, 1997).

Based on our knowledge of the ability of YY1 to interact, either directly or indirectly, with promoter regions for IFN-γ (Ye *et al.*, 1994; Sweetser *et al.*, 1998) and IL-1 (Patten *et al.*, 2000), it may be possible for YY1 to modulate the transcriptional pattern of response to cytokine gene activation, thereby modifying expression of downstream effectors and the apoptotic response. A great deal of evidence exists for a direct relationship between NO and YY1 expression: the endothelial NO synthase (eNOS) promoter possesses a YY1-binding sequence first identified by Karantzoulis-Fegaras *et al.* (1999). Garban and Bonavida (2001) subsequently described inhibition of YY1-binding activity at the Fas promoter in ovarian carcinoma and prostate cancer cell lines in the presence of NO. Further,

a possible mechanism was suggested by Hongo *et al.* (2004) in a PC-3 prostate carcinoma cell line in which introduction of an NO donor was found to inhibit NF-κB and YY1 expression through *s*-nitrosylation. Similarly, Vega *et al.* (2005b) have shown that YY1 repression in Ramos B-cell lymphoma via the chimeric anti-CD 20 mAb, rituximab, results in sensitization to Fas-mediated apoptosis. Thus, there is likely a significant association between YY1 activity and both cytokine and death receptor expression, particularly in tumor systems, with a potential for chemo- and immunoresistance and consequent survival via these pathways.

Role of YY1 in immune resistance
Resistance to Fas. Prostate cancer cell lines have been examined for sensitivity to Fas-induced apoptosis using the FasL agonist monoclonal antibody CH-11. The tumor cells were found to be resistant to CH-11-induced apoptosis. However, treatment of the tumor cells with IFN-γ, NO donors, or some chemotherapeutic drugs sensitized the tumor cells to CH-11-induced apoptosis. Sensitization was accompanied by increased expression of Fas, both at the cell surface and cytosolic. The mechanism of upregulation of Fas gene expression by these sensitizing agents was examined. We hypothesized that sensitization may be the result of de-repression of a transcription repressor. The examination of the Fas promoter revealed a silencer region with a putative binding site for YY1. Experiments were designed to test this hypothesis. We demonstrated that both IFN-γ and NO-releasing reagents treatment inhibited YY1 expression and DNA-binding activity. Further, deletion of the silencer region of the Fas promoter resulted in significant transcriptional activity assessed by a luciferase-based reporter system driven by different variations of the Fas promoter. These studies suggested strongly that YY1 negatively regulates Fas transcription and expression and, in addition, YY1 regulates tumor cell resistance to Fas-induced apoptosis (Garban and Bonavida, 2001). Furthermore, it was shown in prostate carcinoma cell lines that tumor-derived TNF-α regulates Fas resistance and that inhibition of TNF-α sensitized the cells to Fas-induced apoptosis (Huerta-Yepez *et al.*, 2005). Recently, we have also shown that YY1 negatively regulates Fas expression in B-NHL cell lines and also regulates resistance to Fas-induced apoptosis (Vega *et al.*, 2005a, b). We further investigated the mechanism by which IFN-γ and NO inhibited YY1 expression and activity. We demonstrated that IFN-γ induces the expression iNOS and release of NO. NO inhibits both NF-κB and YY1 activity by *s*-nitrosylation (Hongo *et al.*, 2004). Further, inhibition of NF-κB activity resulted in the concurrent inhibition of YY1, suggesting that YY1 transcription is under the regulation of NF-κB (Figure 11).

Resistance to tumor necrosis factor-related apoptosis inducing ligand. Prostate cancer cells were also found to be resistant to tumor necrosis factor-related apoptosis
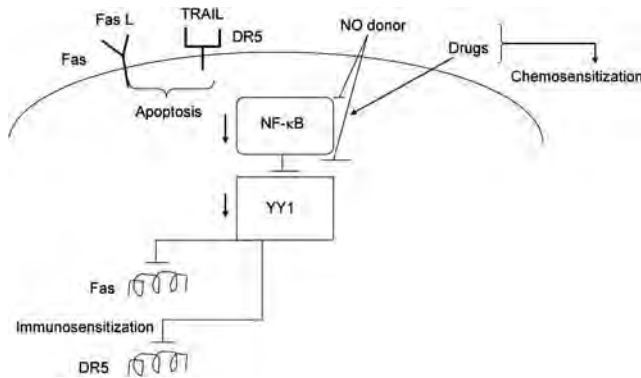
Figure 11 Regulation by YY1 of tumor cell response to apoptotic stimuli. This figure schematically describes the role of YY1 in the regulation of both chemoresistance and immune resistance. Tumor cells constitutively express NF-κB and YY1 activities. It is known that NF-κB is a survival factor and is involved in the transcription regulation of several proapoptotic gene products. Likewise, YY1 has been shown to regulate negatively the expression of Fas and DR5 and hence, regulates resistance to both Fas-ligand and TRAIL-induced apoptosis. Inhibition of NF-κB and/or YY1, for example, by chemical inhibitors or by NO donors, results in the inhibition of NF-κB and YY1 and resulting in both chemosensitization and immunosensitization of drug/immune resistant tumors to apoptosis.

inducing ligand (TRAIL)-induced apoptosis. We examined the mechanism of resistance and demonstrated the treatment of tumor cells with NO or cytotoxic drugs (e.g., cis-diammine dichloroplatinum). CDDP sensitized the tumor cells to TRAIL-induced apoptosis (Huerta-Yepez et al., 2004). The mechanism of sensitization revealed that the tumor cells, following treatment with drug, overexpressed the TRAIL receptor DR5. The upregulation of DR5 was investigated and the findings revealed that the DR5 promoter contains one putative YY1-binding site. Inhibition of YY1 by NO or drugs resulted in inhibition of YY1 expression and activity. Further, using a DR5 reporter system, we demonstrated that deletion of the region containing the YY1-binding site and/or mutation of the YY1 site resulted in significant upregulation of luciferase activity over background level in a reporter system. This finding strongly suggested that YY1 negatively regulates DR5 transcription. The direct role of YY1 in DR5 expression was also corroborated by the use of siRNA YY1, which resulted in upregulation of DR5 expression and sensitization of the cells to TRAIL-induced apoptosis (Huerta-Yepez et al., 2005) (Figure 11).

Role of YY1 in chemoresistance
Prostate cancer cells are resistant to various chemotherapeutic drug-induced apoptosis. However, the cells can be sensitized following treatment with NO. The sensitization resulted in the downregulation of antiapoptotic gene product Bcl-2 and inhibition of both NF-κB and YY1 activity. The role of YY1 in chemosensitization was demonstrated by the use of siRNA YY1 whereby the transfectants were sensitive to CDDP-induced apoptosis in the absence of NO (Huerta-Yepez et al.,

2005). These findings suggested that YY1 regulates drug resistance though the exact mechanism is not yet clear.

YY1 activity and metastatic potential
Beier and Gorogh (2005) provided evidence that downstream targets of YY1 activity are not limited to intracellular signaling. In a series of experiments, they demonstrate that YY1 and AP2 coactivation may potentiate their activity as galactocerebrosidase gene suppressors. Galactocerebrosidase is an enzyme overexpressed upon the cell surface of a variety of cancers. The accumulation of this protein promotes a reduction of cellular adhesion and inhibits apoptosis, leading to cellular proliferation, migration, and prolonged cell survival, all of which may contribute to carcinogenesis and metastasis.

In vivo and clinical evidence for the role of YY1 in the molecular regulation of tumorigenesis
Information gained from in vitro analyses has been applied to translational clinical models of carcinogenesis with tumor progression seen as the predominant effect of YY1 activity. Preclinical and clinical models that have been investigated include epithelial-based tumors (Sitwala et al., 2002; Seligson et al., 2005), hepatocellular carcinoma (Wang et al., 2001; Parija and Das, 2003), and breast cancer (Pilarsky et al., 2004).

Tumor suppression
  YY1 and human papilloma virus. Studies in the elucidation of the regulation of human papilloma virus (HPV) 16 and 18 and the characterization of their roles in the progression of cervical carcinoma by both May et al. (1994) and Dong et al. (1994) implicate YY1-mediated repression when bound to viral oncogene promoters located in the long control region incorporated within the genome of cervical carcinoma cell lines. Likewise, Lichy et al. (1996) found a relatively marked increase in YY1 binding in nontumor lines of HeLa/fibroblast hybrids when compared to malignant cells. Taken together, these data suggest that YY1 acts to suppress tumor progression in HPV-infected cervical epithelial cells.

  YY1 and basal cell carcinoma. Polymorphism studies of the gene encoding expression of glutathione S-transferase (GST) reveal that its variable expression may account for an individual's differential risk of developing malignancies such as colorectal, ovarian, and breast cancer as well as the potential aggressive nature of that tumor. Analysis of human basal cell carcinoma specimens reveals that absence of tumor progression of the GSTM3 genotype may be a result of YY1 repressive activity upon its recognition motif at the GST locus GSTM3*B (Yengi et al., 1996).

  Tumor activation. Extensive evidence for the role of YY1 in the activation of malignant potential has surfaced. Clinical studies by Brankin et al. (1998) reveal that serum elevations of autoantibodies to nucleophos-

min, an estrogen-regulated nucleolar phosphoprotein that suppresses YY1 transcriptional regulating activities, precede clinical evidence of recurrence in breast cancer patients. Interestingly, patients without recurrence demonstrated no change in serum levels. These data suggest that inhibition of nucleophosmin results in relief of repression of YY1 and is associated with clinical progression of breast carcinoma.

Prognostic/diagnostic significance

The above findings on YY1 expression in cancer cells suggested that YY1 overexpression may play an important role in the regulation of tumor cells' sensitivity and resistance to both chemotherapy and immunotherapy. Thus, tumor cells that overexpress YY1 may be selected during therapy and will exhibit drug/immune resistance and continued overexpression may correlate with disease progression and metastases. Initial studies were examined in prostate cancer using tissue microarrays, and YY1 expression and localization were examined by immunohistochemistry. Analysis of the data demonstrated that cancer cells show higher expression of YY1 than normal tissues. In addition, the data demonstrated that there was a subset of patients that can be identified whose YY1 expression predicted tumor recurrences. These studies suggested that YY1 expression may be considered a prognostic marker independent of circulating levels of prostate-specific antigen and other markers in prostate cancer (Seligson et al., 2005). Hence, it would be of interest to examine the prognostic and/or diagnostic significance of YY1 in other cancers.

Conclusion

YY1, a transcription factor that has been progressively characterized over the last 10 years, has become an intensive focus of study due to its ubiquitous nature, highly conserved molecular sequence, and increasingly apparent central role in embryologic development and differentiation as well as basic cellular functions such as replication, proliferation, senescence, and response to genotoxic stimuli.

Although its diverse functions allow for the context-specific paradoxical effects of transcriptional initiation, activation, and repression, the overwhelming evidence of the role of YY1 in tumor biology would support the theory that YY1 functions to promote carcinogenesis and perhaps even confer cells with a mechanism for evading cell death in the face of genotoxic stimuli including chemotherapy and/or immunotherapy (see Figures 10 and 11). Primary mechanisms appear to include perturbations in cellular surveillance systems as

well as modulation of key genes involved in cell cycle regulation and programmed cell death. As enumerated herein, indirect and direct evidence exist to suggest a regulatory role for YY1 in the activation, progression, and/or maintenance of malignancy in multiple tumor models, both in vitro and in vivo. In addition, translational clincopathologic correlates have been documented in studies of human prostate, lymphoma, breast, and HCC tissues to corroborate these laboratory findings. As the clinical models noted herein are further elucidated and additional malignancies are studied, validation via transgenic mouse models with the use of xenografted tumors, inhibition via oligonucleotide testing in vitro, and in vivo oligonucleotide antisense testing, inhibitory RNA applications, and specific chemical inhibition will be necessary to clarify the role of YY1 in each instance and determine the possibility of utilization of the YY1 transcription factor as a target for antitumor therapy and reversal of drug-immune resistance.

Future directions

Diagnostic
As with other characterized protooncogenes, tumor suppressor genes, and tumor markers including p53 (Li-Fraumani Syndrome), RET oncogene (familial and sporadic medullary thyroid cancer), and Her-2/neu (breast cancer), YY1 may eventually serve as a diagnostic genetic marker for tumors proven to demonstrate a predictive pattern of expression in human tissue studies. Furthermore, predictive response to therapy may be extrapolated from these data and used to stratify patients.

Therapeutic
Lastly, as a potential modulator of cellular transformation and development of carcinogenesis, YY1 may serve as a target for cancer therapy with clinical application of therapeutics currently in use in other malignancies, including specific peptide or organic inhibitors, antisense therapies, and silencer RNA. These and other modalities may serve to enhance the spectrum of effective clinically available agents to choose from in the multimodal treatment of difficult tumors.

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Can we develop biomarkers that predict response of cancer patients to immunotherapy?

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Abstract
Primary objective: The primary objective is to delineate the potential utility of cancer biomarkers
that correlate and predict response to immunotherapy in cancer patients who are refractory to
conventional therapeutics. Unlike significant development of biomarkers that predict response
to chemotherapy, very few biomarkers have been developed to predict the response to
immunotherapy.
Main outcomes and results: This article describes briefly the importance of characterizing and
validating biomarkers for immunotherapy. A few examples have been provided, such as the
transcription factor NF-κB, the transcription repressor Yin-Yang 1 (YY1), the pro-apoptotic
gene product (Smac/DIABLO) and the circulating Fas and Fas ligand. These biomarkers have
been determined to be of prognostic significance in different cancers.
Conclusions: Immunotherapy is considered as an alternative therapy in the treatment of cancer
patients who are refractory to chemotherapy/radiation/hormonal therapies. Cross-resistance to
apoptosis develops between cancer cells that are resistant to conventional therapeutics and
immunotherapy. Therefore, it is important to develop biomarkers that will determine patient
response to immunotherapy.

Keywords: *Immunotherapy, YY1, Fas, Smac/DIABLO, NF-κB, biomarkers*

Introduction

Significant advances have been made in the treatment of cancer by chemotherapeutic
drugs, hormonal drugs and radiation. However, the development and/or acquisition of
tumour resistance to such treatments present a major drawback (Patel & Rothenberg
1994). While patients with early and localized tumours respond to standard therapy,
the majority of cancer patients afflicted with advanced metastatic tumours are
unresponsive to further treatments and these patients will eventually succumb to
incurable disease and die. The mechanism of drug resistance is complex and multi-
factorial (reviewed by Pommier et al. 2004). Much of the research efforts today are
focused on searching for alternative therapeutic strategies that are aimed to reverse or

bypass drug-related resistance mechanisms (Tan et al. 2000). Tumour immunotherapy is an ideal therapeutic approach because it offers several advantages over chemo/hormonal/radio-therapies including low organ toxicity and high tumour selectivity. In immunotherapy, the tumour killing agents are derived from the host's own immune system.

Immunotherapeutic strategies under investigation consider that chemo-resistant tumours are sensitive to immunotherapy. It has been assumed that immunotherapy attacks tumour cells using different mechanisms of action and may not be subjected to the drug-resistant mechanism. However, this does not seem to be the case. Despite these proposed advantages over chemotherapy, immunotherapy today still fails to deliver significant curative rates. Spontaneous and drug-resistant tumours remain virtually resistant to immunotherapy in most cancer patients (Sogn 1998).

It is clear to date that both chemotherapy and radiation mediate their cytotoxic effects through apoptosis (Figure 1). Likewise, immune lymphocytes also primarily kill by apoptosis. Thus, both share common mechanisms of killing. It is likely that the mechanism(s) for resistance of cancer cells to chemotherapy would have common or identical features with the developed resistance to apoptotic by other stimuli including immunotherapy. It follows that a strategy for an effective anti-tumour response is to utilize complimentary pro-apoptotic signals to overcome tumour resistance to immune-mediated apoptosis through the use of sensitizing agents (see review by Ng and Bonavida (2002a)). The modification of apoptosis regulatory gene products can be achieved through the use of sensitizing agents, inhibitors, antisense, siRNAs, etc. which, in combination with immunotherapy, could reverse tumour resistance.

In cancer patients, the response to treatment is dictated by many factors. The ability to stratify patients into groups that respond more positively or negatively to a given treatment would be extremely beneficial. Thus, there have been extensive efforts directed towards identifying biomarkers with such properties. Moreover, a well-characterized repertoire of biomarkers would have significant utility at every stage
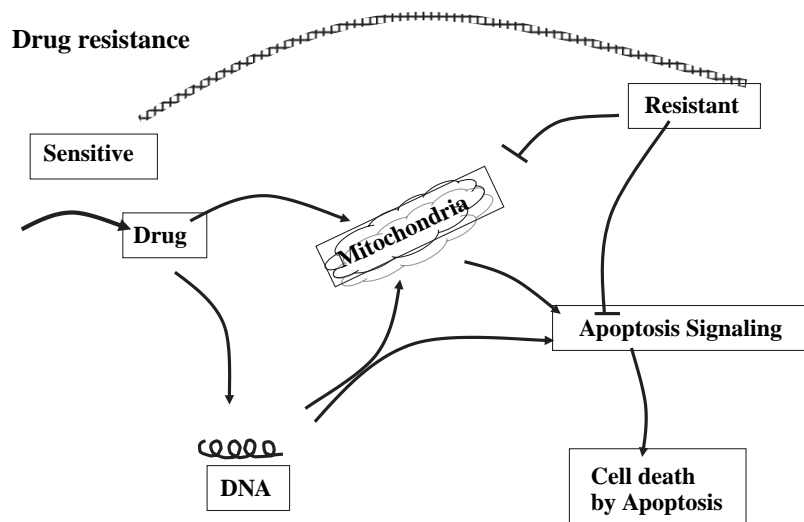

Figure 1. This diagram illustrates that chemotherapeutic drugs have many effects in the cell that culminate in cell death by apoptosis. Resistance to drugs can occur if one or more block interface with the signaling to apoptosis.

of drug development and cancer treatment. In this regard, there are many types of biomarkers, including disease biomarkers (a biomarker that relates to a clinical outcome or measure of disease), staging biomarkers (a biomarker that distinguishes between different stages of disease), efficacy biomarkers (a biomarker that reflects beneficial effects of a given treatment), etc.

Tumour cell sensitization to cytotoxic immunotherapy

Sensitization of tumour cells to cytotoxic immunotherapy involves two complementary signals (Figure 2). The first signal is 'sensitizing' and regulates pro/anti-apoptotic targets, thus facilitating the apoptotic pathway. The second apoptotic signal initiates a partial activation of the apoptotic pathway. The activation is completed by complementation with the first signal.

Identification of gene products that regulate immune resistance: New biomarkers that predict response of failure to respond to immunotherapy

Sensitizing agents that can reverse immune resistance can be used to identify a gene product(s) that regulates resistance. The expression of such gene product(s) in tumour cells may predict clinical response to immunotherapy. Examples of sensitizing agents are presented in Table I.

Studies performed in the laboratory explored several mechanisms of tumour cell resistance to immunotherapy. Figure 3 schematically demonstrates that tumour cells exhibit high basal level of constitutively activated NF-κB and that NF-κB regulates
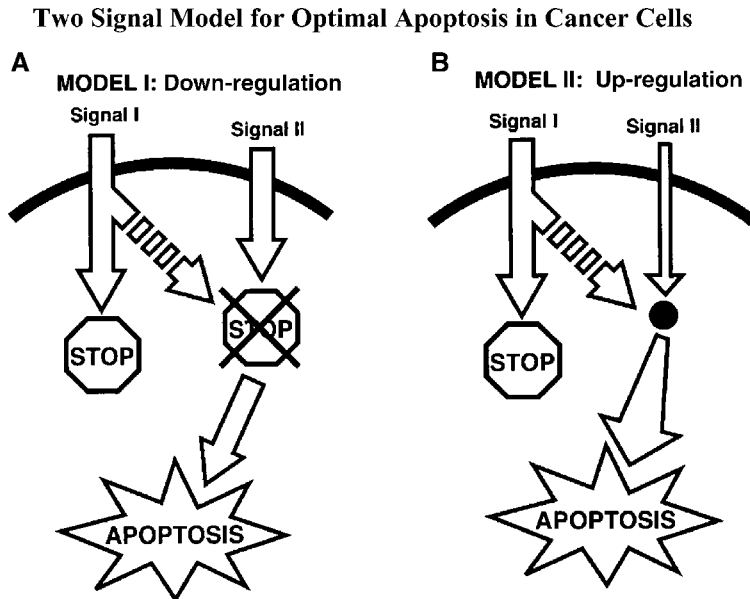

Figure 2. This model proposes that sensitizing agents (Figure 1) can either downregulate (model A) or upregulate (model B) apoptosis regulatory proteins and thus facilitates the cytotoxic agents (signal II) to mediate their apoptotic effects.

Table I. Sensitizing agents and apoptosis induced-stimuli. This table lists examples of sensitizing agents that can reverse resistance of cancer cells to either chemotherapy and/or immunotherapy-induced apoptosis. The apoptosis-inducing immune stimuli are listed and include cytotoxic lymphocytes or members of the TNF-α family.

Sensitizing agents	• Cytotoxic drugs (e.g. CDDP, VP16, ADR, ActD) • Biologic factors (e.g. INF-γ) • Antibodies (e.g. Rituximab (anti-CD20)) • Nitric oxide donors
Apoptosis-inducing cells/factors	• Cytotoxic lymphocytes (CTL, NK) • Recombinant ligands, FasL, TNF-α, TRAIL

many gene products including several anti-apoptotic and inflammatory cytokines. Agents that can inhibit NF-κB can regulate sensitivity to immune-mediated apoptosis (e.g. TNF-α, Fas L, TRAIL) via inhibition of the transcription repressor Ying-Yang-1 (YY1) (Garban & Bonavida 2001). YY1 can be inhibited both by siRNA and NO and its inhibition upregulates the expression of the immune receptors and sensitizes cells to immune-mediate apoptosis.

Studies have identified gene products whose expression regulate tumour cell sensitization to cytotoxic immunotherapy (Table II). These include transcription factors such as NF-κB, YY1, AP-1, anti-apoptotic gene products such as Bcl-2, Bcl-$_{xL}$, XIAP and pro-apoptotic products such as Smac/DIABLO, DR5, RKIP (Huerta-Yepez et al. 2004, Jazirehi et al. 2004, Vega et al. 2004).
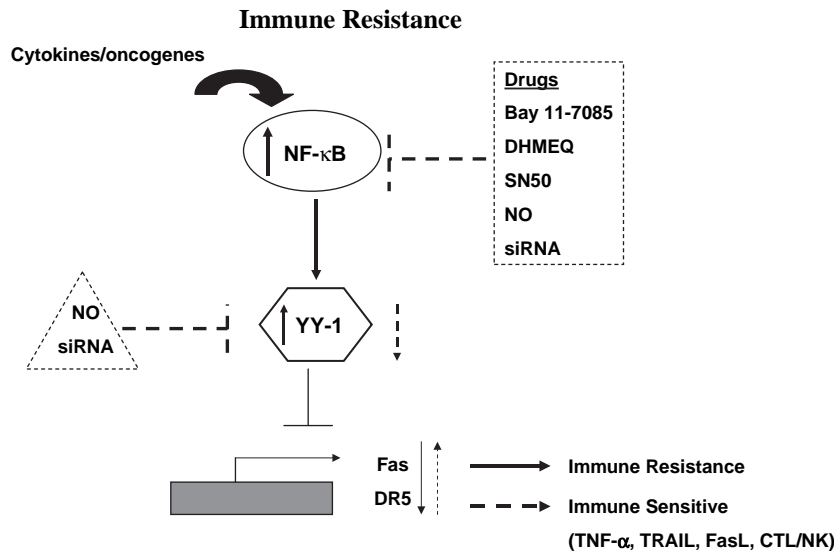

Figure 3. This figure schematically illustrates that tumour cell resistance to immune-mediated apoptosis is the result of several potential mechanisms. The studies have focused on the role of constitutively activated NF-κB in tumour cells and that serves as an anti-apoptotic factor and its inhibition sensitizes the cells to immune-mediated apoptosis. The mechanism by which NF-κB sensitizes tumour cells to apoptosis was examined and was found that YY1 under the transcription regulation of NF-κB plays an important role in the regulation of resistance through the negative regulation of the transcription of immune receptors (TNF-α, FasL and TRAIL). Like inhibition of NF-κB, inhibition of YY1 also sensitizes the cells to immune-mediated apoptosis via upregulation of immune receptors.

Table II. Identification of potential markers for immunotherapy. This table lists a few examples of the underlying mechanisms by which the sensitizing agents function to reverse resistance. The sensitizing agents modify the expression/activity of gene products that regulate resistance. These gene products are potential biomarkers for analysis.

Examples	Biomarkers
• Sensitization to CTL mediated killing	YY1; Bcl-$_{xL}$; Smac/DIABLO, NF-κB,
• Sensitization to Fas/TNF-α/TRAIL-induced apoptosis	AP-1, p38 MAPK
• Sensitization to antibody-mediated apoptosis	Bcl-2; Bcl-$_{xL}$; RKIP; survival pathways

Examples of functional gene products in tumour cell resistance to immunotherapy that might also be useful biomarkers

NF-κB

The NF-κB family of dimeric transcription factors has been shown to modulate cell survival during stress and immune responses (Baeuerle & Baltimore 1996). NF-κB protects cells from apoptosis by promoting expression of survival factors (Wang et al. 1996, 1998). NF-κB also protects cells from immune-mediated apoptosis (Ravi et al. 2001, Huerta-Yepez et al. 2004). Thus, high expression of NF-κB in the nucleus of the tumour may suggest a hyperactivation of anti-apoptotic regulatory gene products and resistance to immune-mediated-apoptosis.

YY1

The transcription repressor (YY1) has been shown to negatively regulate Fas expression in cancer cells and contributes to tumour cell resistance in response to Fas-mediated apoptosis (Garban & Bonavida 2001). Further, recent findings suggest that YY1 also regulates tumour cell resistance to TRAIL-induced apoptosis in prostate cancer cells (Huerta-Yepez et al. unpublished). Finally, YY1 has been shown to be over-expressed in human prostate cancer tissues compared to non-malignant tissue, as measured by tissue micro-array analysis. YY1 expression also appears to have prognostic significance (Seligson et al. 2005). Therefore, the expression of YY1 in tumour tissues may predict response to immunotherapy.

Smac/DIABLO

The TNF ligand super-family plays an important role in the host immune defense against cancer as an anti-tumour death inducing agent (Nagata 1997). This super-family induces cell death by apoptosis in sensitive target cells by the death receptor pathway. The apoptotic signalling pathway is subjected to several levels of inhibition by regulation (Ashkenazi & Dixit 1999). Tumour cells over-express inhibitory and anti-apoptotic proteins (IAPs) (Deveraux et al. 1998) and a mitochondrial molecule, Smac/DIABLO, has been documented to be a neutralizing inhibitor of the anti-apoptotic IAP family of proteins (Du et al. 2000). Thus, tumour cells that express low levels of Smac/DIABLO may be more resistant to immune-mediated apoptosis than cells over-expressing Smac/DIABLO. Indeed, *in vitro*, it is demonstrated that prostate cancer cells resistant to TRAIL can be sensitized by over-expression of Smac/DIABLO (Ng & Bonavida 2002b). In cancer patients, it has recently been

demonstrated that low expression of Smac/DIABLO in renal cancer tumours predicted worse prognosis and survival (Mizutani et al. 2004).

Soluble Fas and soluble Fas ligand

The receptor Fas expressed on the surface of tumour cells can be triggered by the Fas ligand (FasL) expressed on cytotoxic lymphocytes and results in apoptosis of the Fas-sensitive cancer cells (Kagi et al. 1994). While both Fas and FasL are predominately integral membrane proteins, both can also be expressed in soluble, secreted forms. Production of these soluble variants is potentially one survival strategy by tumour cells. At the same time, one may be able to take advantage of this by detecting these products as tumour markers. Soluble Fas (sFas) is generated by alternative mRNA splicing events. As Fas can bind to FasL, Fas secretion may be one of the mechanisms responsible for tumour cell resistance to Fas-mediated apoptosis. Soluble FasL (sFasL) is produced by a different mechanism. Cleavage of membrane-bound FasL by a metalloprotease-like enzyme results in the generation of soluble FasL (Tanaka et al. 1996). Similar to membrane bound FasL, sFasL can also transduce an apoptotic signal in Fas-expressing sensitive cells (Tanaka et al. 1995). However, FasL has been reported to be a weaker inducer of apoptosis compared to membrane bound FasL (Tanaka et al. 1998). Thus, in contrast to membrane FasL, sFasL can protect cells from Fas-mediated apoptosis (Suda et al. 1997). Secreted levels of sFas (Mizutani et al. 1998) and FasL (Mizutani et al. 2001) have been reported to be of prognostic significance in patients with bladder cancer. Further, a combination of serum levels of sFas and sFasL in patients with bladder cancer predicted recurrence after trans-urethral resection (Mizutani et al. 2002). These findings strongly suggest that sFas and sFasL levels can be used as prognostic markers for tumour recurrence
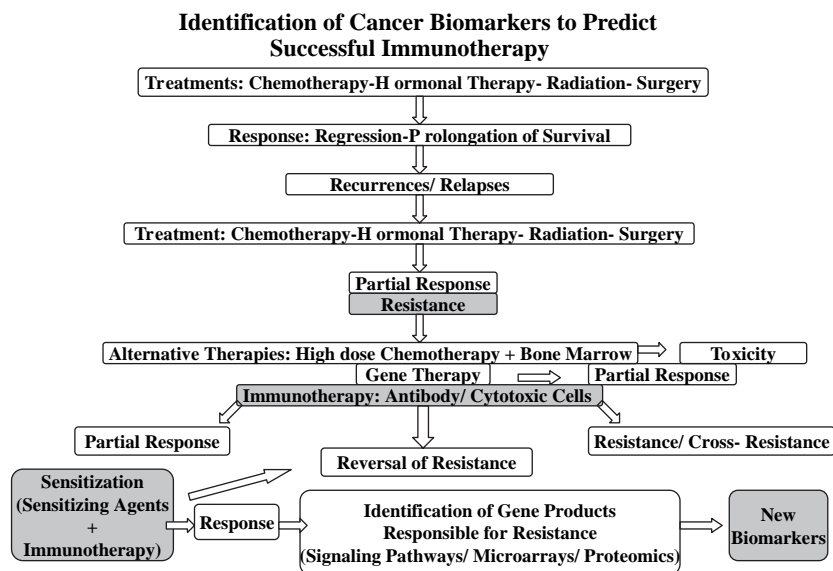

Figure 4. This scheme illustrates the methods used to identify biomarkers of clinical significance for response of resistant cancer cells to immunotherapy.

Concluding remarks

The development of biomarkers for successful immunotherapy is extremely beneficial to stratify the patient population in order to increase their response rate. Oncology is among the first areas to reap benefits of biomarker research, both in terms of diagnosis and treatment. Cancer is often a terminal disease where, if appropriate treatment is not decided quickly, the window of opportunity to treat the disease effectively may be lost. Also, there is increasing number of new oncology medicines and therapeutic choices that can be facilitated by diagnostics to better clarify the type of cancer and choose appropriate treatment. Unlike biomarkers for drug resistance, there have not been many biomarkers for immune resistance and these need to be characterized and validated in the clinical setting. The immune biomarkers examples provided in this report are the first to be completed and to be validated in the clinical setting. A general scheme for the characterization of an immune biomarker is illustrated in Figure 4. While the use of biomarkers in human studies is new, biomarkers are used to help make decisions to select the most promising candidates and/or identify the right patients for particular treatments.

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Principles of Tumor Immunology

BENJAMIN BONAVIDA, Ph.D.

INTRODUCTION

The immune system has evolved to fight microbial infections and the ability to discriminate between self and nonself. The immune system consists of both the innate and the adaptive responses. Innate immunity is the first defense mechanism and consists of both humoral factors and host immune effector cells. The adaptive immune system consists of both the humoral antibody response and the cell-mediated response, both of which are antigen specific and develop memory. While immune surveillance is well documented for microbial infections, it has been controversial for its role in cancer. However, it has been recognized that cancer cells can be recognized by the adaptive immune system via the expression of tumor-associated antigens (TAAs) or tumor-specific antigens. The development of cancer has been explained by the failure of the immune system to be generated or for induction of immune tolerance and escape of tumors from immune elimination. Several lines of evidence, both *in vitro* and *in vivo*, strongly suggest that antitumor cytotoxic lymphocytes or antitumor antibodies can participate in tumor destruction and tumor regression. Therefore, patients who do not respond to conventional therapies (e.g., chemotherapy, hormonal therapy, and radiation) have no alternatives for treatment. Immunotherapy is, therefore, considered a good approach for the treatment of such patients. Several antitumor immunotherapeutic manipulations have been developed and tested experimentally and clinically. Antibody-mediated immunotherapy, by passive administration of monoclonal antibodies (mAbs), has resulted in significant successes in the treatment of various tumors. For instance, several antibodies have been approved by the Food and Drug Adminis-

tration (FDA) for the treatment of non-Hodgkin's lymphoma (NHL) (such as rituximab anti-CD20 mAb), breast cancer (Herceptin anti-Her2/neu mAb), and several other antibodies for the treatment of AML and colon cancer. Cell-mediated immunotherapy has been expanding in an attempt to generate antitumor cytotoxic responses and such strategies include the adoptive transfer of *in vitro* expanded patients' blood leukocytes or tumor-infiltrating lymphocytes (TILs), vaccines to immunize against TAAs, gene therapy, etc. These various strategies have resulted in the proof of principle (i.e., the ability to generate antitumor cytotoxic T lymphocytes *in vitro* and *in vivo* in cancer patients). However, the clinical response has not been optimized and it may be achieved with better manipulations and/or combination chemotherapy and immunotherapy. Furthermore, it is becoming evident that even in the presence of an adequate antitumor cytotoxic response, the resistant cancer cells may develop mechanisms to resist killing by the cytotoxic lymphocytes. In such cases, sensitizing agents may be required to reverse the resistance of the tumor cells to killing and thus facilitate the cytotoxic activity mediated by immunotherapy. Clearly, significant advances have been made in cancer immunotherapy and new approaches will continue to be developed to achieve a clinical response and tumor elimination. Further advances in deciphering the interplay between the tumor and the host immune system and the microenvironment will undoubtedly lead to the development of new classes of immunotherapeutics.

The thymus-derived (T) dependent cell-mediated immune system recognizes tumor cells by virtue of their receptors by recognizing intracellularly derived protein fragments presented on the cell surface by the Major Histocompatibility Complex (MHC) molecules. The T

lymphocytes engage these peptide–MHC complexes through their T-cell receptors. This mechanism allows the immune system to discriminate foreign antigens from self-antigens, as the latter have either induced deletion of self-recognized T cells or developed tolerance. Two types of MHC molecules are involved, namely MHC Class I and MHC Class II. These molecules are displayed on the cell surface and have a peptide-binding domain with a central cleft where it accommodates a peptide sequence. MHC Class I molecules correspond to human leukocyte antigen (HLA)-A, -B, and -C molecules and MHC Class II correspond to HLA-D molecules. Circulating T lymphocytes in lymphoid organs, peripheral blood, and nonlymphoid organs search for this specific MHC–peptide complex, and if found, the cytotoxic cell kills the target. There is a polymorphism of the MHC–peptide interaction and a large repertoire of T-lymphocyte specificities. Auto-reactive T cells that recognize dominant self-antigen are deleted in the thymus in a process called *negative selection*. Also, auto-reactive T cells with weak MHC recognition are neglected. These two processes are known as *central tolerance*. Some T cells escape thymic selection and can recognize self under specific conditions. T cells can recognize nonmutated tumor antigens, which are in fact antiself responses (Pardoll, 1999). T-cell activation requires two distinct signals. Signal 1 is delivered by the interaction between the T-cell receptor (TCR) and antigenic peptides presented on MHC molecules. Signal 2 is provided by one of several nonspecific costimulatory molecules such as CD28 on T cells and B7 family molecules on antigen-presenting cells (APCs).

Usually, cancer cells have Signal 1 only, so they can directly induce tolerance or need to be presented by other types of cells to stimulate the immune system. This is achieved by a process called *cross-presentation*, namely, tumor cells or tumor antigens are taken up by APCs, which process the antigens and present them on the APC cell surface restricted for this on MHC Class I and Class II molecules. APCs such as dendritic cells (DCs) can efficiently prime T cells where they display MHC antigen complexes (Signal 1) together with costimulatory molecules (Signal 2), which activate naïve T cells in a process called *cross-priming*. This process can also cause T-cell unresponsiveness or cross-tolerance (Heath and Carbone, 2001).

Effector Cells

The effector cells of the immune system constitute cells of many types, those with restricted specificities such as CD4 and CD8 and those that are nonrestricted such as natural killer (NK) and NK T cells. The CD4+ T cell's primary role is of a helper type and help APC's activation and maintenance of CD8+ T cells. CD4+ T cells recognize specific peptide sequences presented by MHC Class II. CD8+ T cells are the main effector cells of the adaptive immune response, which mediate antigen-specific MHC-restricted cytotoxic effectors. CD8+ cells recognize peptides presented by MHC Class I molecules through the TCR complex. CD8+ T cells become activated by the TCR–MHC Class I peptide interactions on an APC, together with help from activated CD4+ T cells. This leads to a clonal expansion of antigen-specific cytotoxic T lymphocyte (CTL) that will lyse target cells. Killing by CD8 cells is mediated by the granzyme pathway and the tumor necrosis factor-α (TNF-α) family of ligands and can induce both necrosis and apoptosis.

NK cells are innate effector cells that serve as the first line of defense. They do not express TCRs and are not MHC restricted. However, they express receptors either activating or inhibitory. The NK T cells co-express a TCR and NK 1.1 receptor characteristics of T and NK cells, respectively. They recognize a limited array of peptides and glycolipid antigens presented by the nonpolymorphic MHC-like molecule CD1, which is not widely expressed in most APCs and several tissues (see review by Porcelli and Modlin, 1999).

Auto-Regulation of the Immune System

DCs maintain a balance between immune response and tolerance of tumor antigens. DCs make a decision of what should be presented and recognized as nondangerous self, dangerous self, or nonself (Steinman, 1991; Banchereau and Steinman, 1998). Once T cells are activated, the immune system makes an effort to keep them under control. Lymphocyte expansion after activation and cytokine production are controlled. Cytokine production are balanced by TH1 and TH2 cytokine production. TH1 and TH2 regulate each other. Also, activated T cells undergo apoptosis in the absence of growth factors such as interleukin-2 (IL-2). They also undergo death following antigen recognition by a process called *activation-induced cell death* (Green and Scott, 1994). Another regulatory mechanism is influenced by the costimulatory molecules. CD28 is the ligand for the costimulatory molecules B7.1 (CD80) and B7.2 (CD86), constitutively present on CD4 and CD8 T cells. When these T cells are activated, they upregulate CTLA-4, which is homologous to CD28 but competes and displaces CD28 and attenuates the T-cell response. Cross-linking of B7 with CTLA-4 inhibits T-cell activation. Peripheral T cells with suppressor activity have been the subject of controversy, but a subpopulation of CD4 T cells (5–10%) express CD25 and can suppress auto-reactivity (Shevach, 2001). Tumor cells also develop mechanisms of escape including downregulation of tumor antigen expression or processing, interference with DC presentation, direct inhibition of lymphocyte function, and resistance to apoptosis by immune cells.

The immune system has evolved with various infectious diseases in order to guarantee survival of the host. The immunological effector mechanisms that control microbial

infections (bacterial and viral) are primarily dependent on the routes used for entry by the infectious agents. Diseases that are controlled by utilizing antibodies consist of infectious pathogens that spread in the blood such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, Entoviridae, measles, influenza, and pox viruses (Bachmann and Zinkernagel, 1997; Baumgarth et al., 2000; Ochsenbein, 2002). When the infections target peripheral solid organs with noncytopathic viruses such as hepatitis B and C viruses in humans, these are primarily controlled by the activation of CTLs, which have the ability to extravasate and enter the infected peripheral tissues (Zinkernagel et al., 1996). However, it is well known that both exogenous and endogenous pathogens can illicit both a humoral antibody response and a cell-mediated cytotoxic response. Solid tumors with TAAs may be considered to resemble viral infections of peripheral tissues, so the main effector mechanisms for their control may be through their killing by CTLs (Boon et al., 1997).

IMMUNE SURVEILLANCE OF TUMORS

The immune surveillance theory for tumors was originally proposed by Thomas (1959) and Burnet (1970), and since then, this theory has been challenged by many. The immunological surveillance hypothesis states that tumors arise with similar frequency to infection with pathogens and that the immune system constantly recognizes and rejects these tumors based on the expression of foreign TAAs. The presence of TAAs was based on the finding that tumors induced in animal models were frequently rejected when transplanted into syngeneic hosts, whereas transplants of normal tissues between syngeneic hosts were accepted (Gross, 1943; Foley, 1953; Prehn, 1957). To date, we know that TAAs are the consequences of genetic and epigenetic alterations in cancer cells. A corollary to the original immune surveillance hypothesis is that clinically progressing tumors in all species are not eliminated because they develop active mechanisms of either immune escape or resistance. A fundamental prediction of the immune surveillance hypothesis is that immunodeficient individuals would display a dramatic increase in tumor incidence. This prediction was challenged initially in nude mice whereby there was no increase in spontaneous tumors (Rygaard and Povlsen, 1976; Stutman, 1979). We know that nude mice display an activated NK system that plays an important role in immune surveillance of cancers. Patients with immunodeficiencies revealed a complex pattern of cancer risk (Penn, 1988). There was a significant increase of rare cancers such as Kaposi's sarcoma and lymphoblastic lymphoma, but there was no increase in the common epithelial cancers seen in adulthood (colon cancer, lung cancer, prosthetic cancer, etc.). It became clear that cancers in immunodeficient

individuals are primarily virus associated. Thus, immune surveillance indeed protected individuals against certain pathogen (mostly virus)-associated cancers by either preventing infection or clearing chronic infection by viruses that can lead to cancer. The failure to observe an altered incidence in nonvirus (pathogen-associated cancers) was taken as a strong argument against the classic immune-surveillance hypothesis. A caveat of these studies in the immune-deficient individuals is that they tend not to survive past their 30s or 40s and most non–virus-associated cancers arise late in life. Later studies in mice clearly show that various components of the immune system can indeed modify carcinogen-induced and spontaneous carcinogenesis using mice that had been rendered immunodeficient via genetic knockout (Kaplan et al., 1998; Street et al., 2002). For example, in $RAG^{-/-}$ mice (necessary for immunoglobulin and TCR gene rearrangement), the tumor incidence was not increased at young age; however, at old age (18 months to 2 years), an increase in tumor incidence was observed. Overall, the findings that the distribution of tumors differs in mice with deletions in different immunoregulatory genes suggests that different components of the immune system may modulate carcinogenesis in different tissues. The emerging evidence for immune surveillance systems of carcinogenic events is counterbalanced by evidence that the normal immune response to tumor antigens is tolerance rather than activation.

DO TUMOR CELLS EXPRESS TUMOR-ASSOCIATED ANTIGENS?

Studies using experimental tumors in animals demonstrate that certain tumors (syngeneic and allogeneic) can be rejected by immunological mechanisms and primarily by a cell-mediated immune response. Therefore, much effort has focused on the identification of TAAs that are recognized by human T lymphocytes (Boon et al., 1997; Rosenberg, 1999). Both CD4+ helper and CD8+ cytotoxic T cells recognize antigens presented as small peptides in the groove of surface HLA molecules. CD8+ cells recognize peptides of 8–10 amino acids in length, derived from intracellular cytoplasmic proteins, digested in proteasomes and presented via the endoplasmic reticulum on cell surface Class I HLA molecules. CD4+ cells use a different intracellular pathway and present engulfed proteins digested to peptides in intracellular endosomes and presented on the cell surface of Class II HLA molecules. These different pathways of antigen processing necessitated the development of separate methodologies to identify tumor antigens. These depend on the ability to generate T lymphocytes that can recognize human cancer cells. Several methods were used to identify tumor antigens recognized by CD8+ lymphocytes. These include transfection of complimentary DNA libraries from tumor

cells into target cells expressing the appropriate HLA molecule and then using predetermined antitumor T cells to identify the appropriate transfectants (Boon et al., 1997). Another method consisted of eluting peptides from the surface of human cancer cells and these were pulsed onto APCs and tested for their activity with specific antitumor lymphocytes (Hunt et al., 1992; Cox et al., 1994). The purification and sequencing of these peptides can then lead to the identification of the parent protein. Another technique referred to as "reverse immunology" has been used successfully to identify whether candidate proteins, selected for their overexpression on human cancer cells, represent cancer antigens (Kawashima et al., 1989). The candidate antigen is used to generate *in vitro* CTLs, and if these CTLs recognize intact human cancer cells, the candidate protein is considered a tumor antigen. These findings demonstrated that it was possible to generate human T cells that recognize melanomas, and most tumor antigens recognized thus far have been derived from melanoma. Other antigens expressed on common epithelial tumors have also been identified. A few examples are listed in Table 1.

Because transfection of cDNA libraries into target cells is not effective because the encoded proteins do not travel to the class II pathway, a new technique involving the screening of cDNA libraries fused to a gene encoding invariant chain sequences and designed to generate the transfected protein into the class II pathway has been adopted (Wang et al., 1999a). Many tumor antigens recognized by CD4+ T cells were also identified (Table 1).

Also, cancer associated with viruses can express antigens recognized by T lymphocytes. These include the E6 and E7 antigens on cervical cancers caused by human papillomaviruses, antigens from Epstein–Barr viruses (EBV) on lymphomas and human T-cell lymphotropic virus-1 antigens on adult T-cell leukemia (Lowy and Schiller, 2001). Stoler et al. (1999) have estimated thousands (~11,000) of genomic alterations in cancer cells, and these represent candidates for cancer antigens recognized by the immune system. Also, cancers of the hematopoietic system express unique antigens not shared by other cancers such as the unique clonotype on B-cell lymphomas.

IMMUNE RESPONSE TO CANCER

Are Tumors Immunogenic in Humans?

Studies in mice have shown that adoptive immunotherapy of lymphocytes from immunized donors were able to transfer antitumor immunity in the recipient (Klein and Sjogren, 1960; Old et al., 1962). In humans, adoptive transfer from tumor-bearing patients of lymphokine-activated killer (LAK) cells with non-HLA restriction were able to recognize and kill cancer cells *in vitro* and some clinical response *in vivo* (Rosenberg et al., 1987). In patients with

TABLE 1 Human Cancer Antigens

Type of antigen	References
Class I—restricted antigens recognized by CD8+ lymphocytes	
A. Melanoma-melanocyte differentiation antigens	
MART-1 (Melan A)	Kawakami et al., 1994a
gp-100 (pmell-17)	Kawakami et al., 1994b
Tyrosinase	Brichard et al., 1993
Tyrosinase related protein-1	Wang et al., 1995
Tyrosinase related protein-2	Wang et al., 1996
Melanocyte stimulatory hormone receptor	Salazar-Onfray et al., 1997
B. Cancer-testis antigens	
MAGE-1	van der Bruggen et al., 1991
MAGE-2	Visseren et al., 1997
MAGE-3	Gaugler et al., 1994
MAGE-12	Panelli et al., 2000
BAGE	Boel et al., 1995
GAGE	Van den Eynde et al., 1995
NY-ESO-1	Jager et al., 1998
C. Mutated antigens	
β-Catenin	Robbins et al., 1996
MUM-1	Chiari et al., 1999
CDK-4	Wolfel et al., 1995
Caspase 8	Mandruzzato et al., 1997
HLA-A2R17d	Brandle et al., 1996
D. Nonmutated shared antigen overexpression in cancers	
α-Fetoprotein	Butterfield et al., 1999
Telomerase catalytic protein	Vonderheide et al., 1999
MUC-1	Jerome et al., 1991
Carcinoembryonic apoptosis	Tsang et al., 1995
p53	Theobald et al., 1995
Her-2/neu	Ioannides et al., 1993
Class II–restricted antigens recognized by CD4+ T lymphocytes	
A. Epitopes for nonmutated protein	
gp-100	Li et al., 1998
MAGE-1	Chaux et al., 1999
MAGE-3	Chaux et al., 1999
Tyrosinase	Topalian et al., 1994
NY-ESO-1	Zeng et al., 2000
B. Epitopes from mutated proteins	
CDC 27	Wang et al., 1999a
LDLR-FUT	Wang et al., 1999b
Triosephosphate isomerase	Pieper et al., 1999

Source: This partial list was derived from Rosenberg (2001).

melanoma, it was possible to obtain TILs and these were grown in large numbers *in vitro* and were capable of specifically recognizing cancer antigens in about one-third of patients with melanoma (Muul et al., 1987). TILs adoptively transferred into melanoma patients with IL-2 resulted in an objective response rate in ~35% of patients (Rosenberg et al., 1988). The identification of cancer antigens and their ability to generate an antitumor response was pivotal as it opened the field of cancer immunotherapy and the develop-

ment of various strategies to generate antitumor cytotoxic responses (see later discussion). The immunogenicity of tumor cells, however, does not explain the failure of the immune system to mount an antitumor response in cancer patients and the seldom demonstration of tumor-specific cytotoxic lymphocytes in cancer patients. If one accepts that cancers are immunogenic, then the failure to generate an immune response must be explained by mechanisms by which the tumor evades the host immune system. Several mechanisms have been reported and a few are discussed in the following sections.

Tumor Escape from Immunological Responsiveness and Underlying Mechanisms

The lack of clinical responses points to deciphering the mechanisms of tumor escape. Escape mechanisms include loss of antigen expression by the tumor, the local presence of immunosuppressive factors or cells, and the failure of the tumor to activate antitumor responses.

Antigen Expression

Lymphocytes, macrophages, and APCs perform their effector functions as single cells, but to activate CTLs, they need to interact and collaborate in organized lymphoid tissues. These anatomical structures determine the localization of antigen, cytokines, and bystander contacts through costimulatory molecules. The lymph nodes and spleen provide the milieu necessary for lymphoid cell interactions and activation. CTLs respond to antigen that becomes transiently presented within the organized lymphoid tissues for at least 3–5 days. In contrast, T cells do not react against antigens that are continuously present in lymphoid organs (Webb et al., 1990; Moskophidis et al., 1993). Antigen that is continuously present in secondary lymphoid organs would activate and delete all T cells specific for that antigen. The process of activation followed by physical deletion of the T cells is coined *exhaustion*. In summary, (1) a low antigen dose in lymphoid organs is not sufficient to induce a CTL response, and as a consequence, antigens that strictly stay outside lymphoid organs are immunologically ignored, (2) a sufficient antigen dose over a sufficiently long time in lymphoid organs induces a specific CTL response, and (3) a higher amount of antigen continuously present in lymphoid organs tolerizes the specific CTL response (Ochsenbein, 2002). T-cell activation requires two distinct signals. Signal 1 is delivered by the interaction between the TCR and antigenic peptides presented on MHC molecules. The second signal is provided by at least one of several antigen non-specific costimulatory molecules including the interaction of CD28 on T cells with B7 family molecules on professional APCs (Matzinger, 1994; Chambers and Allison, 1997). Signal 1 alone has been correlated with induction of T-cell energy or deletion. Experimentally, tumors expressing costimulatory molecules are usually rejected more efficiently than control tumors. *In vitro* experiments indicate that DCs process exogenous cell debris and present the peptides on MHC Class I. This cross-priming process may be even more efficient for apoptotic cells than for necrotic cells. Cross-priming has also been shown in *in vivo* experiments (Bevan, 1976). The lack of relevant cross-priming may explain why most peripheral solid tumors do not induce an efficient antitumor response. The T-cell activation state induced by cross-presentation is thought to depend on multiple factors such as T-cell frequency, antigen levels, the maturation stage of DCs presenting the antigen, or the presence of potent CD4+ T-cell help (Spiotto et al., 2003). Increasing data implicate the mechanism of immune deviation as a means by which tumors escape an immune intervention. Mosmann et al. (1986) have shown that fully differentiated T-cell clones exhibited one or two distinct cytokine profiles. TH1 clones produce interferon-γ (IFN-γ) and IL-2, whereas TH2 clones produce IL-4, IL-5, and IL-10. The TH1 response seems to be of primary importance in the cell-mediated immune response that is important for antitumor immunity. Tumors may provoke immune deviation by stimulating a TH2 response.

Downregulation of the antigen-processing machinery and particularly the MHC Class I pathway has been documented in a large variety of human tumors (see review Marincola et al., 2000). Global MHC Class I loss may be due to mutations in combination with deletion of β2-microglobulin genes. Down-modulation of MHC Class I genes can result from down-modulation of the transporter associated with antigen presentation (TAP) genes and components of the immune proteasome (Restifo et al., 1996; Seliger et al., 1996). Other mechanisms of immune resistance mechanisms by tumors include the expression of secreted or cell surface molecules that either kill or inhibit cellular components of the effector immune response, for example, transforming growth factor-β (TGF-β) (Moretti et al., 1997).

IMMUNOTHERAPY

Given the failure of conventional therapeutics to treat resistant metastatic and recurrent cancers and the belief that cancers can respond to the immune system, much attention has been focused on the development of cancer immunotherapy. Several approaches have been considered to improve the immune response to tumors, including peptide vaccines, recombinant viral vaccines, recombinant bacterial vaccines, nucleic acid vaccines, DC vaccines, and the use of heat shock proteins as adjuvant (Pardoll, 1999). Also, adaptive cellular immunotherapy has been considered. Animal models have explored various costimulatory strategies (Hurwitz et al., 2000).

It has been postulated that efficient therapeutic vaccination should aim at triggering naturally occurring specific

T-cell responses to destroy tumor cells. However, there remains a discrepancy between successful findings in animals and the clinical success rate in cancer patients. The identification of tumor antigens and the generation of tumor-reactive lymphocytes are necessary but not sufficient to treatment efficacy. There is evidence for circulating naïve or antigen-specific tumor-reactive T cells in cancer patients (Romero et al., 1998). The mere presence of these cells has fueled hope for the development of therapeutic vaccines. Such experimental vaccination has been shown to induce specific immunity in a considerable number of cancer patients. These responses, however, only sporadically induced tumor regression in patients with metastatic diseases and rarely documented remission and survival (Cranmer et al., 2004). Tumors have also developed mechanisms to escape immune destruction. For instance, tumor cells are embedded in stroma, a network of extracellular matrix that harbors inflammatory cells such as macrophages, granulocytes, and DCs. Such cells produce factors that promote tumorigenesis and angiogenesis and contribute to immune evasion (Ganss et al., 2004). There is a basic assumption that activated tumor-reactive T cells are capable of migrating and destroying the tumor tissue. However, this was not the case in experimental animal models (Ganss et al., 2004). Angiogenesis is a component of the tumor phenotype, is essential for nutrient delivery, and has been neglected in large part by tumor immunologists. Leukocyte extravasation is tightly controlled by blood vessels and contributes to tumors' intrinsic resistance to infiltration. Therefore, effective immune responses require both fully armed effector cells and a tumor microenvironment permissive to infiltration and destruction (Ganss et al., 1999).

A compelling body of evidence argues that vascular components of the tumor stroma, not tumor cells, are indeed the primary targets during immune-mediated tumor rejection. For example, studies by the Blanheister group have reported that the rejection of transplantable tumors by CD4 and CD8 effectors was not mediated by direct tumor cell killing but was correlated with the ability to secrete IFN-γ, which in turn modulates IFN-γ receptor–positive stroma and inhibits angiogenesis (Qin and Blankenstein, 2000; Qin et al., 2003). It appears that angio-immunotherapy is a primary strategy, whereas inflammatory stimuli such as irradiation normalize the vasculature and activate endothelin, thereby promoting effector cell infiltration and antigen-driven tumor cell elimination.

Cell-Mediated Immunotherapy

1. *Cellular adoptive immunotherapy*: Several approaches have been considered for the use and/or activation of antitumor cytotoxic lymphocytes. The ability to successfully immunize patients against defined cancer antigens has facilitated the generation of antitumor T cells that can be expanded and used for adoptive immunotherapy (Rosenberg, 2001). There is also the potential to clone lymphocytes derived from a single cell selected for the high avidity to tumor antigen and to grow them in large numbers for passive adoptive immunotherapy. Also, genetic modification of lymphocytes can be performed to improve their antitumor efficacy and such studies are under active investigation. Peripheral blood mononuclear cells (PBMCs) stimulated *in vitro* with IL-2 generate LAK cells. Adoptive transfer of these cells shows promise in clinical studies but is mostly disappointing. The same findings were also observed with TILs. Expansion and cloning of lymphocytes derived from a single antigen-specific T cell and adoptive transfer after myeloablative conditioning chemotherapy resulted in cell proliferation and persistent clonal repopulation and correlated with tumor regression in patients with melanoma (Dudley et al., 2002).

2. *Cancer vaccines* (Ribas et al., 2003): Whole-cell tumor vaccines with whole-cell tumor lysates have been under clinical investigations for decades. Allogeneic or autologous tumor cells are processed and injected with a powerful adjuvant to attract host APCs. Randomized clinical trials have not been able to reflect an antitumor activity (Mitchell, 1998; Sondak et al., 2002).

3. *Gene-modified tumor vaccines*: These consist of autologous tumor cells stably transfected with an immunostimulating gene like cytokine (Dranoff, 1998). These have been tested in clinical trials (Soiffer et al., 1998).

4. *Peptide-based vaccine*: Tumor-derived peptide epitopes that contain the appropriate HLA-restricted amino acid sequence can be synthetically manufactured and administered with an immune adjuvant. This has led to an enhanced immune response (Rosenberg et al., 1998a).

5. *Naked DNA*: Intramuscular injection of naked DNA sequences results in gene expression and the generation of an immune response. Naked DNA plasmids have low immunological potency for generating antitumor responses to self-antigens in humans (Rosenberg, 2001).

6. *Viral vectors*: A variety of gene therapy vectors have been adapted to cancer immunotherapy. This application has resulted in weak immunological responses in humans (Rosenberg et al., 1998b); however, vectors that carry tumor antigens and costimulatory adhesion with other immune-enhancing molecules are being tested (Abrams and Schlom, 2000).

7. *Prime boost strategy*: The sequential administration of naked DNA and a viral vector has resulted in synergistic immune activation for tumor antigens (Ramshaw and Ramsay, 2000).

8. *Bacterial vectors*: Tumor gene segments have been introduced into bacteria such as *Salmonella* and *Listeria*, resulting in protected immunity in animals (Pan et al., 1995).
9. Ex vivo *APC-based vaccines*:
   a. DCs: The ability to generate large quantities of DCs in culture from hematopoietic precursors or peripheral blood allowed extensive testing in clinical trials (Timmerman and Levy, 1999). Immunizations in humans with antigen-loaded DCs resulted in detectable T-cell activation to tumor antigens with occasional clinical responses.
   b. *Immunocytokines*: Local delivery of cytokines to tumors to provide high paracrine levels in order to set a sequence of events leading to recognition of surface molecules on cancer cells (Imboden et al., 2001).
10. *Blockade of tumor-derived suppressor factors*: In the presence of activation of cytotoxic T cells, factors derived from the tumor or the microenvironment can prevent tumor killing. For instance, blocking the immunosuppressive prostaglandin E_2 (Huang et al., 1998) or TGF-β (Fakhrai et al., 1996) resulted in enhancing antitumor response.
11. *Vaccines in clinical trials* (Mocellin et al., 2004): Whole-cell vaccines (autologous or allogeneic): The efficacy of autologous or allogeneic whole-cell vaccines has not been confirmed by Phase III trials done in the therapeutic or adjuvant settings.
    a. *Tumor lysates*: Phase III studies done in the therapeutic or adjuvant setting have not confirmed the efficacy of lysate vaccines (Sondak and Sosman, 2003).
    b. Heat shock proteins: There have been encouraging results in humans, as heat shock proteins have been tested in Phase III clinical trials in patients with renal cell carcinoma or with melanoma.
    c. *Antigen-defined vaccines*: There has been little clinical benefit thus far.
    d. *DC vaccines*: There is strong preclinical evidence for the use of DCs in humans for antitumor vaccination, and the results of clinical trials have been conflicting, with a lack of objective tumor response in 12 of 35 trials. A comparative Phase III trial of DC vaccination of patients with advanced cancer is underway.

Antibody-Targeted Immunotherapy (van de Loosdrecht et al., 2004)

There have been successes in the application of engineered antibodies in the treatment of both hematological malignancies and solid tumors. Two basic strategies have been considered, namely, active versus passive immunization. Active immunotherapy refers to the induction of a specific immune response to malignant cells *in vivo*. Passive immunotherapy refers to therapeutic interventions with antibodies that recognize tumor-specific antigens. The development of therapeutic antibodies considers several factors for success, that is, immunogenicity, the nature of the antibody, selection of the appropriate antibody for optimal binding with human effector cells, and selection of target antigens both for induction of apoptosis and/or for regulation of tumor growth (Chinn et al., 2003). Immunogenicity of immune antibodies was initially one of the most striking limitations of using therapeutic antibodies because of the rapid generation of human antimouse antibodies, which prevented the repeated use of the antibody. New recombinant technologies have been developed to reduce the immunogenicity of genetically engineered chimeric antibody in which the antigen-binding region is of murine origin and the remaining sequence of human origin. Humanized antibodies have also been developed. Higher affinity binding to target antigen is a key feature of the antibody, which influences the dose for optimal antigen saturation and efficacy. The ability both to activate host effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) and to induce apoptosis or inhibit survival is also required.

Selection of appropriate antigens for antibody-targeted therapy and the level of antigen expression on neoplastic cells are of significant importance for optimal efficacy. The expression of lineage-specific differentiation antigens in hematological malignancies (e.g., CD20 in B-cell NHL and CD33 in AML) provides unique targets for antibody-targeted therapy. Stable irreversible binding between antibody and antigen should be obtained to maximize host effector cell activity and internalization of antigen by the antibody. Antibody-targeted therapy can be included in an induction phase, during intensification as consolidation therapy with or without autologous or allo-PSCT or as maintenance therapy in a postremission phase of treatment. In addition, antibody-targeted therapy can be included in a treatment program in relapsing diseases or in patients with primary refractory disease.

The expression of the lineage-specific differentiation antigen CD20 has provided a unique target (Chinn et al., 2003). The absence of CD20 on stem cells allows the recovery of normal B cells following treatment, which lends to the destruction of malignant and normal B cells. The absence of CD20 on plasma cells results in only a slight decrease of immunoglobulin. The chimeric anti-CD20 mAb (rituximab; Rituxan) is approved by the FDA for the treatment of B-NHL alone or in combination with chemotherapy (Smith, 2004; Jazirehi and Bonavida, 2005). CD22 is a B-cell–restricted sialo-glycoprotein present in virtually all developing B cells but is detectable on the cell surface only at the mature stages of differentiation. Antibody-targeted therapy with anti-CD22 (Epratuzumab), a humanized

immunoglobulin G1 (IgG1) antibody, has been administered in patients with NHL (Cesano and Gayko, 2003). CD33 is a cell surface glycoprotein receptor that is specific for myeloid cells. CD33 is expressed on ~90% of leukemia blasts and leukemic myeloid precursor cells, as well as on normal myeloid precursor cells, but not on CD34+ pluripotent stem cells (van Der Velden et al., 2001). CD33 is internalized after binding with anti-CD33 antibody and is not therapeutically useful. A clinically successful approach used anti-CD33 antibody targeted in combination with an immunotoxin, gemtuzumab ozogamicin (Mylotarg) (Bross et al., 2001). The anti-CD52 mAb CAMPATH-1 is used in patients with CLL given extensive prior therapy (Witzig et al., 1999). In some hematological malignancies, edrecolomab (Panorex) has been approved in Europe for colorectal cancer and trastuzumab (Herceptin) for the treatment of breast cancer in the United States (White et al., 2001). In 2004, the FDA approved bevacizumab anti-vascular endothelial growth factor (VEGF) mAb as first-line therapy for metastatic colorectal cancer (Ferrara et al., 2004). Radioimmunotherapy has been developed in NHL (Chesen, 2002). The most widely used in patients with NHL are directed against CD20 linked to either [131]I (tositumomab, Bexxar) or [90]Y (ibritumomab, Zevalin).

FUTURE CONSIDERATIONS FOR SUCCESSFUL ANTICANCER IMMUNOTHERAPY

The success of immune-mediated therapies in leukemia and lymphoma and certain solid tumors has stimulated translational research and the development of more effective immunotherapeutic strategies. Promising new approaches will combine classic chemotherapy with tumor-specific targeted immunotherapy. The more we learn about the natural relationship between the endogenous immune system and tumors as they develop, the more effective methods will be developed to manipulate those responses for successful therapeutic antitumor effects. The development of cross-resistance between chemotherapy and immunotherapy requires rethinking about new therapeutic approaches to treat drug-resistant tumor cells. There seems to be the need to develop strategies to overcome tumor cell resistance to apoptotic stimuli by the use, for example, of sensitizing agents and their clinical use in combination with immunotherapy for successful therapeutic results. Thus, treatment of malignant tumors requires at least two complimentary signals for functional complementation using a nontoxic sensitizing agent (Signal 1) that alters the cellular signaling pathways and the expression profile of the apoptotic associated molecules and, hence, facilitates the cytotoxic activity of the therapeutic agent (e.g., chemotherapeutic drugs, radiation, immune cytotoxics) (Signal 2) for

the induction of apoptosis (Ng and Bonavida, 2002). Understanding why the endogenous and exogenous immune responses fail to control tumorigenesis is pivotal to improving antitumor immunotherapy. The concept of antigen dose, tumor localization, induction of tolerance, and immunological ignorance must be investigated to decipher the underlying mechanisms that regulate antitumor responses. A vaccine efficiently delivering tumor antigens to secondary lymphoid organs may lead to tumor control, and eradication of the tumor load is neither too great nor too difficult to reach. Only continuous stimulation of endogenous hosts or *in vitro* expanded and adoptively transferred effector T cells will likely be sufficient to lead to rejection of tumors quickly enough before the selection of escaped mutants. Although tumor vaccines have not been clinically efficient *in vivo*, adoptive cell therapy with the expanded *in vitro* tumor-specific effector cells has advantages such as manipulating the host before the autologous cells are transferred, for example, by eliminating host lymphocytes with suppressor activity, thus providing the transfer cells an optimal environment for anticancer therapy.

The realization that immunotherapy may be in large part due to its activity to tumor endothelial cells and the concept of "angio-immunotherapy" are promising, although inflammatory stimuli normalizing the vasculature also activate endothelia, thereby promoting effector cell infiltration and antigen-driven tumor cell elimination. Thus, for future consideration, it is pivotal to understand the complex cascade of events that is needed to induce angiogenesis and permit effector cells entry into tumors.

It seems likely that the next decade will witness significant advances in the basic science of tumor immunology. The tools available are numerous, including the sequence of the human genome, the development of microarray technologies, the generation of knockout and transgenic animals for studies and *in vivo* imaging techniques, etc. All of these will facilitate the tasks of tumor immunologists. The challenge, though, lies in the clinical application of such knowledge. Clearly, the anticancer potential of adaptive immunity has not been fully exploited. The observation that patients will develop an immunological response to vaccination and they are more likely to benefit from the treatment than those who do not develop such a response raises a challenge to identify the conditions and their underlying basis for the differential response and thus apply them to a larger set of patients. To this aim, multi-parametric comparison of the dynamic immunological variables in patients who respond and those who do not respond by use of high-throughput biotechnology (e.g., DNA microarrays and proteomics) might be useful in the identification of molecular pathways leading to immune rejection in cancer. A better understanding between innate and adaptive immune responses, the discovery of mechanisms underlying immunological tolerance, and acknowledgment of the importance of both cell-

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mediated and humoral-adaptive immunity for the control of tumor growth are leading to more comprehensive immuno-therapeutic approaches that take into consideration the many variables that define an effective antitumor response.

Acknowledgments

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IN PREPARATION

The transcription repressor YY1 negatively regulates DR5 expression and controls cancer cells resistance to TRAIL-induced apoptosis: Reversal of resistance by inhibitors of YY1

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Running Title: Negative regulation of DR5 transcription by YY1

Keywords: YY1, TRAIL, DR5 transcription, resistance

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Abbreviations

CaP: prostate cancer

DETANONOate: (Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1,

2-diolate

DHMEQ- dehydroxymethylepoxyquinomicin

DHT: 5-α dihydrotestosterone

DR: death receptor

DTT: 1,4-dithiothreitol

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

NF-κB: nuclear factor κB

NO: nitric oxide

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

RIPA: radioimmunoprecipitation assay (buffer)

SDS: sodium dodecyl sulfate

TPA: 12-O-tetradecanoylphorbolacetate

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand.

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

SiRNA: small interfering RNA.

Abstract

Most tumors are resistant to TRAIL and need to be sensitized to undergo apoptosis. We have recently reported that TRAIL-resistant human prostate carcinoma cell lines can be sensitized by various NF-κB inhibitors (Huerta-Yepez *et al.,* 2004), and sensitization correlated with upregulation of DR5 expression. We hypothesized that a gene product(s) regulated by NF-κB with DR5 repressor activity may be responsible for the DR5 regulation. Inhibition of NF-κB activity resulted in significant upregulation of DR5 expression and sensitized prostate tumor cells to TRAIL-mediated apoptosis and synergy is achieved. Treatment of PC-3 cells with NO inhibited both NF-κB and YY1 DNA-binding activity and also inhibited YY1 expression. Treatment of PC-3 cells with YY1 siRNA resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The direct role of YY1 in the regulation of DR5 expression was examined in an DR5 luciferase reporter system (pDR5). Two constructs were generated, the pDR5/-605 construct with a deletion in the promoter region containing the putative YY1 DNA-binding region (-1224 to -605) and a construct pDR5-YY1 with a mutation of the YY1 DNA-binding site. Transfection of PC-3 cells with these two constructs resulted in significant (3-fold) augmentation of luciferase activity over baseline suggesting the repressor activity of YY1. The present findings demonstrate that YY1 negatively regulates DR5 transcription and expression and hence, regulates resistance to TRAIL-induced apoptosis. Inhibitors of YY1 expression and/or activity in combination with TRAIL may be useful in the treatment of TRAIL-resistant tumor cells.

Introduction

Conventional anti-tumor therapies consist primarily of chemotherapy, radiation, hormonal therapy and immunotherapy. Such treatments result in significant clinical responses. However, many patients experience relapses and the tumors become refractory to such therapeutics. Alternative therapies have been considered that include immunotherapy, both antibody and cell-mediated, with potential anti-tumor activity (Martinet *et al*., 2002; Xu *et al*., 2004; Senba *et al.,* 1998). Antibody-mediated therapies have been applied clinically in the treatment of lymphoid and non-lymphoid tumors (Blattman and Greenberg, 2004; Robak, 2004; Murillo *et al.,* 2003). There is considerable effort to generate anti-tumor CTL in an effort to overcome drug resistance (Chung *et al.,* 2004; Dermime *et al.,* 2004). Since cytotoxic lymphocytes and cytotoxic antibodies mediate their killing by various mechanisms, including the TNF-α family (TNF-α, Fas ligand and TRAIL) (Shresta *et al.,* 1998), several studies have also considered such soluble recombinant ligands or agonist antibodies for anti-tumor therapies (Shresta *et al.,* 1998). TNF-α and Fas ligand have been shown to be toxic to normal tissues, however, TRAIL is minimally toxic to normal tissues and is selectively toxic to transformed tumor cells (Walczak *et al.,* 1999; Ashkenazi and Dixit, 1999; Lawrence *et al.,* 2001). TRAIL is a type II transmembrane protein of the TNF-α family (Wiley *et al.,* 1995) and forms homotrimers that bind three receptor molecules (Hymowitz *et al.,* 1999). Functional studies showed that this ligand triggers apoptosis in a variety of tumor cell lines but not most normal cells, implicating its potential therapeutic application in cancer treatment (Schmaltz *et al.,* 2002; Sayers *et al.,* 2003; Ashkenazi and Dixit, 1999). TRAIL induces apoptosis through interaction with its receptors. Four

homologous human receptors for TRAIL have been identified, including: DR4, DR5, DCR1, DCR2 as well as a fifth soluble receptor called osteoprotegerin (OPG) (MacFarlane *et al.,* 1997; Walczak *et al.,* 1997; Pan *et al.,* 1997; Sheridan *et al.,* 1997). Both the death receptors DR4 and DR5 contain a conserved death domain (DD) motif and signal apoptosis. The other two receptors appear to act as decoys as they can inhibit TRAIL-induced apoptosis when overexpressed. Studies *in vitro* and *in vivo* have demonstrated that TRAIL exerts anti-tumor activity (Walczak *et al.,* 1999). Recently, agonist antibodies against TRAIL receptors DR4 and DR5 are being clinically tested in humans (Buchsbaum *et al.*, 2003). Although many tumors are sensitive to TRAIL-mediated apoptosis, the majority, however, are resistant. Resistance can be overcome by the use of sensitizing agents that modify the apoptosis signaling pathways, and thus facilitating the apoptotic effect of TRAIL. Several sensitizing agents have been reported by us as well as others in a variety of tumor cell models (Yamanaka *et al.,* 2000; Ng *et al*., 2002; Jazirehi *et al.,* 2001; Huerta-Yepez *et al*., 2004; Tillman *et al*., 2003; Schmelz *et al.* 2004). These studies revealed that the development of tumor cell resistance to TRAIL is multi-factorial and is dependent on the cell tumor system used.

Previous findings indicated that normal tissue resistance to TRAIL might have been due to the expression of decoy receptors DR1 and DR2 and these compete with the death receptors DR4 and DR5 (Sheridan *et al*., 1997). However, further studies indicated that this paradigm is not generalized and that many tumor cells express both decoy and death receptors and that other mechanisms, like the downstream signaling events of the receptors, may be involved in the regulation of resistance and sensitivity to TRAIL (Aggarwal *et al.,* 2004; LeBlanc and Ashkenazi, 2003).

Several studies have revealed that several sensitizing agents upregulate DR5 and DR4 expression which correlated with sensitivity to TRAIL (Shigeno *et al.,* 2003; LaVallee *et al*., 2003; Johnston *et al*., 2003). However, the mechanisms by which the receptors are upregulated by these agents and the regulation of DR5 expression in TRAIL-resistant cells have not been studied. The regulation of DR5 expression has been reported by using a pDR5-reported system. In this study, the authors have demonstrated that SP1 is a major transcription factor that regulates DR5 expression (Yoshida *et al*., 2001).

YY1 is a 414 amino acid Kruppel-related zinc transcription factor that binds to the CG (A/CC) CATNTT consensus DNA element located in promoters and enhancers of many cellular and virus genes. YY1 physically interacts with and recruits histone-acetyl-transferase, histone-deacetylase and histone-methyl-transferase enzymes to the chromatin and may thus direct histone-acetylation, deacetylation and methylation at YY1 activated or repressed promoters (Coull *et al.,* 2000). In previous findings, we have reported that Fas expression is negatively regulated by the transcription repressor Yin Yang 1 (YY1) through binding of YY1 to the silencer region of the Fas promoter (Garban and Bonavida, 2001). We thereby examined the DR5 promoter region and identified a putative YY1 binding site (-804 to –794 bp) (Yoshida *et al*., 2001). Thus, we hypothesized that the upregulation of DR5 by certain sensitizing agents, such as the nitric oxide donor DETANONOate, to TRAIL-induced apoptosis (Huerta-Yepez *et al*., 2004) may be due to inhibition of both NF-κB and YY1 activities. This hypothesis was tested in this study and the followings were examined: (1) Does treatment of prostate cancer cells with DETANONOate sensitize the tumor cells to TRAIL-induced apoptosis? (2) Does

DETANONoate treatment upregulate DR5 expression? (3) Does DETANONOate inhibit NF-kB and YY1 DNA-binding activities? (4) Does YY1 negatively regulate DR5 transcription and expression as determined by a) treatment of tumor cells with YY1 siRNA and b) by deletion of a YY1 containing region in the DR5 promoter or mutation of the YY1 binding site in the promoter, and (5) does inhibition of YY1 activity by siRNA sensitize the cells TRAIL-induced apoptosis?

Results

Mechanisms by which DETANONOate sensitize CaP cells to TRAIL-induced apoptosis

We have recently reported that the NO donor DETANONOate sensitizes tumor cells to TRAIL-induced apoptosis (Huerta-Yepez *et al.,* 2004), though the exact mechanism is not known. This study investigates the biochemical mechanism of sensitization of CaP cells to TRAIL apoptosis. Four CaP cell lines, namely, the androgen-dependent LNCaP and the androgen-independent PC-3, CL-1 and DU-145, were treated with pre-determined optimal concentrations of TRAIL (5 ng/ml), DETANONOate (1000 μM) or combination for 18 h. The cells were then analyzed for apoptosis by flow cytometry for the presence of activated caspase 3 as described in methods. The findings demonstrate that all 4 cell lines were relatively resistant to treatment with TRAIL or DETANONOate as single agents, whereas the combination resulted in significant potentiation of apoptosis in all 4 cell lines (Figure 1A). The potentiation of apoptosis was synergistic as determined by isobologram analysis (Figure 1B). The PC-3 cell line was selected as representative for the subsequent experiments.

A. Upregulation of DR5 expression by DETANONOate

Several reports demonstrated that sensitization of tumor cells to TRAIL-induced apoptosis by various agents correlated with the upregulation of DR5 expression (Wang and El-Deiry, 2003(a); Shigeno *et al.,* 2003; LaVallee *et al.,* 2003; Nakata *et al.,* 2004). Hence, we examined whether DETANONOate-induced sensitization to TRAIL-induced apoptosis also correlated with the upregulation of DR5 expression in CaP cells. PC-3 cells were treated with DETANONOate (1000 μM for 18 h) and the cells were examined for DR5 expression. Treatment with DETANONOate significantly upregulated surface DR5 expression as determined by flow cytometry as there was a significant increase in the mean fluorescence intensity (MFI) (Figure 2A). RT-PCR analysis demonstrated a significant increase of DR5 transcription by DETANONOate compared to control GAPDH (Figure 2B Top Panel). In addition, DR5 total protein expression was significantly increased by DETANONOate as determined by western (Figure 2B Bottom panel). Altogether, these findings demonstrate that DETANONOate upregulates both surface and total DR5 protein expression.

B. Inhibition of NF-κB activity and both YY1 activity and expression by DETANONOate

Our recent findings have demonstrated that NO inhibits NF-κB activity in PC-3 cells (Huerta-Yepez *et al.,* 2004). The transcription repressor YY1 negatively regulates Fas expression through its binding to the silencer region of the Fas promoter (Garban and Bonavida, 2001). By analogy, we examined the DR5 promoter (Yoshida *et al*., 2001) and detected a putative YY1 DNA-binding site. We postulated that inhibition of NF-κB by DETANONOate will also inhibit YY1 repressor activity and will result in the

upregulation of DR5. We analyzed the effect of DETANONOate on NF-κB and YY1 DNA-binding activities. Treatment of PC-3 cells with DETANONOate significantly inhibited NF-κB DNA-binding activity as determined by EMSA. The inhibition by 1000 uM DETANONOate was more significant than that by 500 uM DETANONOate (Figure 3A). Similarly, DETANONOate inhibited YY1 DNA-binding activity and the inhibition was also dependent on the concentration of DETANONOate used (Figure 3B Top Panel). In addition, treatment with DETANONOate inhibited YY1 expression as determined by western (Figure 3B Bottom Panel). These findings demonstrate that DETANONOate inhibits both NF-κB activity and YY1 DNA-binding activity and suggests that inhibition of YY1 by DETANONOate may be responsible, in part, for the observed (Figure 2) upregulation of DR5 expression.

Regulation of DR5 expression by YY1

The above finding suggested that YY1 may be involved in the negative regulation of DR5 transcription. Experiments were designed to directly demonstrate the role of YY1 in the regulation of DR5 expression and sensitivity to TRAIL-apoptosis. We first examined the effect of YY1 siRNA. Transfection of PC-3 cells with YY1 siRNA, but not with control siRNA, resulted in inhibition of YY1 transcription (Figure 4A), significant upregulation of DR5 surface expression (Figure 4B) and the cells were significantly more sensitive to TRAIL-induced apoptosis compared to cells transfected with siRNA negative control or untreated control cells (Figure 4C). These findings demonstrate that YY1 plays a role in both the negative regulation of DR5 expression and also in tumor cell resistance to TRAIL-induced apoptosis.

The direct role of YY1 in DR5 transcription was examined using a DR5 luciferase reporter system, pDR5 (Yoshida *et al*., 2001). PC-3 cells transfected with the full-length pDR5 promoter showed baseline luciferase activity (Figure 5). In order to determine if YY1 repressor activity is involved in DR5 transcription, we used a pDR5 construct in which the putative YY1 DNA-binding region (-804 to –794 bp) was deleted (pDR5/-605) as described in methods. PC-3 cells transfected with pDR5/-605 resulted in upregulation (3 fold increase) of luciferase activity compared to cells transfected with pDR5 (Figure 5A) suggesting that the deleted YY1-conforming region was responsible for inhibition of transcription. To directly show that YY1 is responsible for the negative transcriptional regulation of DR5, we prepared a construct of the DR5 reporter system in which the YY1 DNA-binding site was mutated as described in methods. Cells transfected with the pDR5-YY1 mutant showed a significant increase in luciferase activity compared to cells transfected with the wild type reporter pDR5 (Figure 5A) and the luciferase activity was comparable to cells transfected with pDR5/-605.

The role of NF-κB in the regulation of DR5 expression via YY1 was corroborated by the use of the NF-κB inhibitor DHMEQ (Ariga *et al.,* 2002). Treatment of PC-3 cells transfected with pDR5 with DHMEQ resulted in augmentation of luciferase activity compared to untreated cells (Figure 5B). The PC-3 cells transfected with pDR5- YY1 mutant showed significant augmentation of luciferase activity as compared to pDR5 transfected cells, and treatment with DHMEQ did not have any additional effect (Figure 5B). These findings suggest that DHMEQ inhibition of NF-κB resulted in significant inhibition of YY1 and the regulation of DR5 by NF-κB was primarily due to YY1 repressor activity. These findings demonstrate that YY1 negatively regulates DR5

transcription and that YY1 is the dominant transcription repressor factor in the pDR5/-605 construct.

Discussion

This study presents evidence for the first time for the role of the transcription repressor YY1 in the regulation of DR5 expression in tumor cells and its role in the resistance to TRAIL-mediated apoptosis. Prostate cancer cell lines, used as model system, are resistant to TRAIL-induced apoptosis. However, these cell lines become sensitive to TRAIL following treatment of the cells with inhibitors of YY1 expression and/or activity. Inhibition of YY1 resulted in the upregulation of DR5 expression and sensitization of the cells to TRAIL-induced apoptosis. Inhibition of YY1 by DETANONOate or by YY1 siRNA in PC-3 cells resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The direct role of YY1 in the negative regulation of DR5 expression was demonstrated by using a pDR5 reporter system in which the YY1 binding site was either deleted or mutated and transfectants resulted in significant augmentation of luciferase activity over baseline of cells transfected with the full promoter. These findings suggest that tumor cells can escape killing by TRAIL via constitutive overexpression of YY1, which in turn negatively regulates DR5 expression and sensitivity to TRAIL. These findings also suggest that agents that can inhibit YY1 transcription, expression or activity may be suitable for their use in the treatment of TRAIL-resistant tumor cells when used in combination with TRAIL or an agonist DR5 antibody.

TRAIL selectively induces apoptosis in a variety of TRAIL sensitive tumor cells and it has been shown not to be cytotoxic to the majority of normal tissues (Ashkenazi *et al.,* 1999). Therefore, TRAIL and agonist antibody to DR5 are currently being examined clinically *in vivo* as potential cancer therapeutics. The apoptotic anti-DR5 monoclonal antibody is a promising agent for cancer treatment (Ichikawa *et al.,* 2001; Ohtsuka *et al.,* 2003). These strategies are based on the expression of functional death receptors on cancer cells. However, many cancer cells are resistant to TRAIL due to dysregulation of the apoptotic signaling pathways. For example, resistance to TRAIL was shown in neuroblastoma cells to be due to the lack of expression of caspase 8 and caspase 10. In addition, there was dysregulation of signal adaptors and activation of inhibitory molecules (Eggert *et al.,* 2000). There was a correlation between levels of the cellular FLICE inhibitory protein (c-FLIPs), with sequence homology to caspases 8 and 10, and TRAIL resistance (Griffith *et al.,* 1998; Kim *et al.,* 2000). Bax inactivation in MMR deficient tumors caused resistance to TRAIL (Burns and El-Deiry, 2001; LeBlanc *et al.,* 2002). Levels of Smac/DIABLO also conferred resistance to TRAIL (Ng and Bonavida, 2002; Fulda *et al.,* 2002). Therefore, agents that can sensitize tumor cells to TRAIL apoptosis are sought. Overexpression of DR5 in TRAIL resistant tumor cells restores TRAIL sensitivity (Yeh *et al.,* 1998; Kuang *et al.,* 2000; Mitsiades *et al.,* 2001). DR5 expression in a number of JURKAT clones was highly correlated with sensitivity to TRAIL (Jang *et al.,* 2003). These findings provide evidence for a strategy to induce DR5 expression in order to enhance the susceptibility of cancer cells to TRAIL or anti-DR5 monoclonal antibody-induced apoptosis. Our findings here support this contention and

demonstrate that inhibition of YY1 resulted in upregulation of DR5 expression and sensitization to TRAIL apoptosis.

It is not clear why certain tumors express low levels of DR5 and what regulates DR5 expression in cancer cells. The low expression of DR5 in tumor cells and its upregulation by inhibitors of NF-κB prompted us to examine the possible role of transcription repressors under the regulation of NF-κB that result in the upregulation of DR5 expression. Previous findings demonstrated that Fas expression was under the negative regulation of NF-κB via a transcription repressor YY1 (Garban and Bonavida, 2001). We examined the possible role of YY1 in the negative regulation of DR5 following transfection of PC-3 with the DR5 promoter, which has a putative YY1 DNA-binding site. Our findings implicate the role of YY1 in the regulation of DR5 by various lines of evidence. We demonstrate that treatment of CaP cells with the NO donor DETANONOate, which we have reported to inhibit both NF-κB and YY1 DNA-binding activity (Huerta-Yepez *et al*., 2004; Hongo *et al.,* unpublished), resulted in upregulation of DR5 expression and sensitization of the cells to TRAIL-induced apoptosis. The direct role of YY1 in the negative regulation of DR5 was shown in experiments in which cells transfected with YY1 siRNA, but not with control siRNA, resulted in upregulation of DR5 and sensitization to TRAIL. Further, transfection of PC-3 cells with a luciferase DR5 reporter system in which a deletion in the promoter that contains the YY1 binding site (pDR5/-605) or direct mutation of the YY1 site (pDR5-YY1 mutant), resulted in significant augmentation of luciferase activity over baseline activity in cells transfected with the wildtype reporter pDR5. DR5 transcription has been examined by Yoshida *et al.,* (2001) using a reporter system for human DR5 and demonstrated that transient

transfection with several 5 prime deletion mutants identified the minimal promoter element sparing –198 to –116. Two SP1 sites were found responsible for the basal transcription activity of the DR5 gene promoter. Our results here demonstrate that DR5 expression can be negatively regulated by YY1. In addition to YY1, other mechanisms in the negative regulation of DR5 may also be involved. Recently, Nakata *et al.,* 2004 reported that histone deacetylase inhibitors upregulate DR5 expression. HDACs activated DR5 transcription through its promoter activation in a p53 independent manner. Also, HDAC inhibitors sensitized the cells to TRAIL-induced apoptosis. HDAC's upregulate transcription of certain genes through the inhibition of HPAG and subsequent changes in the chromatin structure (Kouzarides, 1999; Strahl and Allis, 2000).

Several inducers of DR5 have been reported. For example, p53 has been reported to trans-activate DR5 gene expression (Wu *et al.,* 1997, 1999; Takimoto and El-Deiry, 2000). In addition, genotoxic agents like doxorubicin, etoposide, gamma radiation also induce expression of DR5 in a p53-dependent or independent manner (Sheikh *et al.,* 1998; Gibson *et al.,* 2000; Gong and Almasan, 2000; Nagane *et al.,* 2000; Wen *et al.,* 2000). Preliminary findings demonstrate that CDDP-induced upregulation of DR5 expression in CaP cells is due, in part, to inhibition of YY1 activity (Baritaki *et al.,* unpublished). Dexamethasone and interferon gamma induce apoptosis and DR5 expression in cell lines with mutant p53 (Meng and El-Deiry, 2001). Other agents such as sulindac-sulfide (Huang *et al.,* 2001; Tang *et al.,* 2002; He *et al.,* 2002) and 2-methoxy-estradiol (LaVallee *et al.,* 2003) have been reported to be strong inducers of DR5. However, the mechanisms of these inducers are poorly understood. It will be of interest to determine whether YY1 is implicated in some of these above studies.

The negative regulation of DR5 by YY1 reported here is reminiscent of other studies in which YY1 negatively regulates gene transcription. Recently, Sui *et al.,* 2004 reported that loss of YY1 resulted in a significant increase in the level p53. The augmentation of p53 by ablation of YY1 resulted in the induction of p53 ubiquitination *in vivo*. In that study, the function of YY1 is independent of its transcription activity. YY1 activates and represses transcription by the interaction with cellular transcription factors, namely TVP, TAF, TF2B and SP1 (Austen *et al.,* 1997; Seto *et al.,* 1993; Lee *et al.,* 1993; Chiang and Roeder, 1995; Usheva and Shenk, 1994).

The NF-κB transcription factor family consists of several structurally-related proteins such as c-Rel, Rel-A, Rel-B, p50/p105, p52/p100 which form homo or heterodimers with each other and regulate the expression of a number of genes (Barkett and Gilmore, 1999). Shigeno *et al.,* (2003) show that the hut-7 cells exhibited binding activity of NF-κB composed of a p50 homodimer without any stimulation and TRAIL-induced inhibition of NF-κB binding by Rel-A/p50 heterodimers. Rel-A/p50 appears to play a role in resistance to TRAIL (Ravi *et al.,* 2001). Interferon-α pre-treatment inhibits Rel-A/p50 NF-κB in cells sensitized to TRAIL-induced apoptosis. The mechanism of interferon-induced upregulation of DR5 and inhibition of c-Rel-A-p50 NF-κB activity was not examined at the transcription level. It is possible that c-Rel-A-p50 regulates YY1 expression and its inhibition by interferon-α or by DETANONOate, shown to inhibit p50, may explain the upregulation of DR5. The role of NF-κB in the regulation of DR5 expression and TRAIL-induced apoptosis was reported by Ravi *et al.,* (2001). These studies show that NF-κB induced the expression of both death receptors DR4 and DR5. The c-Rel subunit of NF-κB transcription factor induces expression of DR4 and DR5.

Conversely, a trans-dominant mutant of the inhibitory protein IκB-α, or a transactivation deficient mutant of c-Rel reduces expression of either death receptor. These studies, however, did not address directly the role of NF-κB in the transcription regulation of DR5.

The role of DETANONOate in the inhibition of NF-κB and sensitization to TRAIL-apoptosis is in agreement with studies by Chawla-Sarkar *et al.,* (2003) who showed that the NO donor nitrosylcobalmin (NO-Cbl) sensitized tumor cells to TRAIL-induced apoptosis. NO-Cbl inhibits IKK activity by decreasing phosphorylation of IκB-α and inhibition of NF-κB DNA-binding activity and confirmed by transfection of an NF-κB-driven luciferase reporter system. Further, NO-Cbl was shown to increase expression of DR5 and DR5 mRNA (Bauer *et al.,* 2002). In this study, the regulation of DR4 and DR5 transcription by NF-κB was not shown and may be through inactivation of YY1 as reported here. Yoshida *et al.,* (2001) reported the promoter structure and transcription initiation sites of the TRAIL receptor DR5. The nuclear factor-kappaB (NF-κB binding sites) lies between +385 and +394 in intron 1. It is possible that NF-κB activates DR5 expression via these binding sites in intron 1. In our present findings we demonstrate that NF-κB inhibition by DHMEQ affects only the YY1 deletion mutant using a reporter system not containing intron 1. A recent study (Nakata *et al.,* 2004) demonstrates that histone deacytelase inhibitors (HDCAI) upregulate DR5 expression and sensitize cells to TRAIL apoptosis.

When compared to non-transformed cells, cancer cells are more sensitive to TRAIL-induced apoptosis following exposure to TRAIL treatment (Ashkenazi *et al.,* 1999). Apoptosis induction in response to most DNA-damaging drugs usually requires

the function of the tumor suppressor p53, which engages primarily the intrinsic type of the apoptotic signaling pathway. However, many tumor cells exhibit inactivated and/or mutated p53 and thus resist chemotherapy (Gasco and Crook, 2003; Soussi, 2003). TRAIL induces apoptosis in cancer cells regardless of the p53 status, and thus can reverse resistance to chemotherapy (Wang and El-Deiry, 2003(b)). The combination of chemotherapy with TRAIL has been found to be effective in killing cancer cells with wildtype p53, presumably through the induction of DR5 expression (Nagane *et al.,* 2001; Wang and El-Deiry, 2003(a)). *In vitro,* prior exposure of Bax deficient cells to topoisomerase inhibitors such as CPT-11 and VP-16 restores TRAIL sensitivity mainly by upregulation DR5. Knocking down p53 targets, DR5 and Bak, which are most likely involved in sensitizing Bax deficient human cancer cells to TRAIL with small molecules such as siRNA, showed that silencing DR5 in Bax deficient cells significantly inhibited TRAIL sensitivity. P53-dependent upregulation of DR5 contributed significantly to restoration of TRAIL sensitivity in Bax deficient cells upon DNA damage. These studies suggest the usefulness to identify small molecules that can reverse TRAIL resistance in cancer cells containing mitochondrial apoptotic defects as well as p53 mutations. Targeting of DR5 in cancer cells might be a useful therapeutic strategy. The agents so far available to upregulate DR5 expression are largely those that activate p53. Efforts to identify agents to upregulate DR5 expression independently of p53 may be useful in TRAIL-based cancer therapies. In this study, we have identified YY1 as a factor that negatively regulates DR5 expression independent of p53 since the PC-3 tumor cells are deficient in p53 and inhibition of YY1 sensitizes the cells to TRAIL-induced apoptosis

by chemicals such as DETANONOate, siRNA or inhibition of its transcription (e.g. NF-κB inhibitors) (See Figure 6- schematic diagram).

We have recently reported that prostate cancer tissues overexpress YY1 compared to normal prostate epithelial cells. This was accomplished by immunohistochemistry using human prostate cancer tissue microarrays (Seligson *et al.,* unpublished). Based on the present findings, we suggest that overexpression of YY1 in cancer tissues may negatively regulate the expression of the TNF-α family and thus, governs tumor cells' resistance to host immune effector cells and/or immunotherapy. In addition, YY1 overexpression may be implicated in the pathogenesis of human cancer. The present findings suggest that agents that upregulate DR5 expression, such as targeting YY1 to inhibit its expression and/or activity, and in combination with other therapies should result in the reversal of tumor cell resistance to TRAIL-induced apoptosis.

Materials and methods

Cells and culture conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent LNCaP and the androgen-independent CL-1 (LNCaP-derived) (Tso et al., 2000) cell lines were kindly provided by Dr Arie Belldegrun at UCLA. Cells were maintained as a monolayer in 80 mm2 plates in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Life Technologies) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and

1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA). FBS was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/l R1881 methyltrienolone (New Life Science Products, Boston, MA, USA). The cell cultures were incubated at 37°C and 5% carbon dioxide.

Reagents

The anti-DR5 and anti-β-actin monoclonal antibodies were purchased from Biosource International (Camarrillo, CA, USA) and from Calbiochem (San Francisco, CA, USA), respectively. The human recombinant TRAIL was obtained from PeproTech Inc. (Rocky Hills, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-active caspase 3 and FITC-conjugated IgG were purchased from PharMingen (San Diego, CA, USA). The DETANONOate was obtained from Alexis (San Diego, CA, USA). The SureSilencingTM siRNA kit was purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). The QuickChange Site-Directed Mutagenesis kit was obtained from Stratagene (La Jolla, CA. USA). DHMEQ was derived from Dr. Umezawa, Keio University, Tokyo, Japan (Ariga *et al.,* 2002).

Plasmid construction.

The pDR5 WT promoter luciferase (pDR5 promoter) reporter plasmid and the pDR5 promoter with the 5'-deletion mutant –605 that includes the YY1 binding site (pDR5/-605) have been previously characterized (Yoshida, et al, 2001). The pDR5 plasmid missing the YY1 binding sequence (pDR5-YY1 mutant) was generated by using

the QuikChange site-directed mutagenesis method (Stratagene). The mutagenesis reaction contained the pDR5 plasmid as a template DNA and two complementary oligonucleotides, each containing the desired mutation surrounded by 15 bp of flanking sequence on both the 5' and the 3' sides. A PCR-based method used the complementary primers pDR5-yy1 F (5'-TGT CATG TACTGGGACTACAGGCC-3') and pDR5-yy1 R (5'-GGGAGGCTGAGGTGGGAGTATCTGC-3'). The PCRs contained 125 ng of each primer, 1X PFU buffer [20 mM Tris-HCl (pH 8.8), 10 mM (NH4)2SO4, 2 mM MgSO4, 100 μg of bovine serum albumin/ml, 0.1% Triton X-100], a 2.5 μM concentration of each deoxynucleoside triphosphate, and *Pfu* polymerase. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 45 seg, 69°C for 1 min, and 72°C for 11 min. PCR products were purified by QIAquick PCR purification kit QIAGEN Inc (Valencia, CA. USA).

Luciferase DR5 promoter reporter assay

PC-3 cells were transfected by electroporation using pulses at 250 V/975 mF (Bio-Rad), with 20 μg of pDR5 promoter, pDR5-YY1 mutant or pDR5/-605. After transfection, the cells were allowed to recover overnight and were cultured in six-well plates. Cells were treated or left untreated with the NO donor DETANONOate (1000 μM) for 18 h. Cells were then harvested in 1X lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate. Data were normalized by protein concentration using Bio-Rad protein assay.

Cell treatments

Log-phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6×10^4 cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1, and PC-3 cells were synchronized by treatment with 1% FBS for 18 h prior to each experiment. The treatment of LNCAP cells was in a medium with 1% of serum and the treatments of DU145, CL1, and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate, or the combination for 18 h.

Flow cytometry

To examine the expression of DR5 on the surface of PC-3 cells, flow cytometric analysis was performed. PC-3 cells were detached with PBS-EDTA (1 mM), washed with PBS, resuspended in PBS containing 10% of human normal serum and incubated for 1 h at room temperature. Cells were washed with PBS containing 0.5% BSA and resuspended in PBS- 0.5 % BSA and incubated with the anti-DR5 monoclonal antibody (Mab) at room temperature for 45 min. Cells were washed twice with PBS-0.5% BSA. PE-conjugated goat anti-mouse IgG Caltag (Burlingame, CA, USA) was added and incubated at room temperature for 30 min. Cells were washed again in PBS-0.5% BSA and fixed with 1% paraformaldehyde. Flow-cytometric analysis was performed using EPICSR XL-MCL (Coulter, Co. Miami, FL, USA), with the System II^{TM} Software and the mean fluorescence intensity was recorded.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice-cold 1X phosphate-buffered saline (PBS) and were resuspended in 100 µl of the cytofix/cytoperm solution (PharMigen, San Diego, CA, USA) for 20 min. Thereafter, the samples were washed twice with ice-cold 1X perm/wash buffer solution (PharMingen) and were stained with FITC-labeled anti-active caspase 3 mAb for 30 min (light protected). The samples were subsequently washed once with 1X perm/wash buffer solution and 250 ml of 1X PBS were added prior to flow cytometry analysis on a flow cytometer EPICSR XL-MCL (Coulter, Co. Miami, FL, USA), with the System II^{TM} Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Western blot analysis

PC-3 cells were cultured at a low FBS concentration (1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with DETANONOate (1000 mM). The cells were then lysed at 4°C in RIPA buffer (50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN, USA). Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). An aliquot of total protein lysate was diluted in an equal volume of 2 X SDS sample buffer, 6.2mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 5 min.

The cell lysates (40 μg) were then electrophoresed on 12% SDS–PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi et al., 2001). Levels of β-actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) using the public domain NIH Image J Program (also available via the internet).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA of PC-3 cells was extracted and purified from ~1X10^6 cells for each experimental condition by a single-step monophasic solution of phenol and guanidine isothiocyanate-chloroform using Trizol® reagent (Life Technologies, Inc.). 3 μg of total RNA was reverse-transcribed to first strand cDNA for 1 h at 42°C with SuperScript^{TM} II reverse transcriptase (Life Tchnologies, Inc) in a 20 μL reaction and performed per the manufacturer's specifications using random primers. Amplification of 1/10 of these cDNA by PCR was performed using the gene-specific primers of DR5. Internal control for equal cDNA loading in each reaction was assessed using the following gene specific glyceraldehydes-3-phosphate dehydrogenase (GAPDH). PCR amplification of each DNA sequence was carried out by the "Hot Start" method using Titanium Taq™ polymerase (Clontech) with the following one-step thermal cycling incubation: 95°C/30 s, 68°C/1 min for 30 (DR5) or 25 (GAPDH) cycles, with a final extension at 68°C/3 min. The number of cycles was established based on preliminary titration of the relative amount of amplified product for each gene representing the linear phase of amplification process. The amplified products were resolved on 1.5% agarose gel electrophoresis and their

relative concentrations were assessed by densitometric analysis of digitized ethidium bromide-stained image, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

Nuclear extracts preparation

Nuclear extract preparations were carried out as previously (Garban and Bonavida, 2001). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA, USA). After washing, cells were lysed in 1ml of NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl2, and 0.5% NP-40) on ice for 5 min. Samples were centrifuged at 300 g at 4° C for 5 min. The pellet was washed twice in NP-40 buffer. Nuclei was then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN, USA). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at -80°C.

EMSA

Nuclear proteins (5 µg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB or YY1 using EMSA Kit Panomicst (Panomics Inc., Redwood City, CA, USA) following the manufacturer's instructions and as described previously (Vega et al., 2004). 10 µl was subjected to 5% polyacrylamide gel

electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-Nþ (Amersham Pharmacia Biotech,Germany) using the Trans-Blots SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3 min. The detection was carried out as per the manufacturer's instructions, after the membranes were exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The Relative concentrations were assessed by densitometric analysis as mentioned above.

siRNA Transfections

PC-3 cells were cultured in 1 ml of RPMI medium supplemented with 5% FBS. Transfections were performed by using lipofectamine 2000 CD Reagent supplied by Invitrogen (Life Technologies, Carlsbad, CA, USA) and the SureSilencing^{TM} siRNA kit supplied by SuperArray Bioscience Corporation (Fredrick, MD) according to the manufacturers' instructions. Briefly, 3 μl of YY1 siRNA or a negative control of siRNA solution were incubated with 4 μl of the transfection reagent in serum-free RPMI medium 1640 for 25 min to facilitate complex formation. The resulting mixture was added to PC-3 cells cultured in a 24-well plate with 1 ml of medium. To determine the extracellular expression of the DR5 receptor, the cells were harvested 36 hours after transfection and stained with anti-DR5 monoclonal antibody for 30 min then anti-mouse IgG-PE for 20 min. The expression was then analyzed by flow cytometry. To determine the PC-3 sensitization to TRAIL-mediated apoptosis, 24 h after transfection the cells were treated for 18 hrs with TRAIL (1 and 2.5 ng/ml) and fixed and permeabilized for anti-active

caspase-3-FITC antibody staining. The cells were then analyzed by flow cytometry under the same conditions described above.

Isobologram analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/NO combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5, and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 mM) as described (Berenbaum, 1978). Combinations yielding a cytotoxicity of 30-75% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose–response curves using active caspase 3 analysis for LNCaP, DU145, CL-1, and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18 h.

Statistical analysis

The experimental values were expressed as the mean ± s.d. for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's t-test was used for comparison between two groups. Significant differences were considered for probabilities < 5% ($P < 0.05$).

Acknowledgements

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Figure Legends

Figure 1. DETANONOate sensitizes CaP cell lines to TRAILmediated apoptosis. (A) The CaP cell lines LNCaP, DU145, CL-1, and PC-3 were treated with TRAIL (5 ng/ml) in the presence or absence of DETANONOate (1000 μM) for 18 h. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analyzed by flow cytometry as described in methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. *$p<0.05$, **$p<0.02$. (B) This figure establishes synergy as determined by isobologram analysis.

Figure 2. DETANONOate induces upregulation of DR5 expression. (A) Upregulation of DR5 surface expression by DETANONOate. PC-3 cells were treated with 1000 μM DETANONOate for 18 h and surface expression was performed with anti-DR5 mAb as described in methods. The data represent the observed mean fluorescence intensity (MFI) and are the mean of three independent experiments. *$P< 0.05$, medium *vs* cells treated. (B) Top panel: Upregulation of mRNA expression of DR5 in PC-3 cells by DETANONOate. Untreated or cells treated with 1000 μM of DETANONOate for 18 hr were used to isolate total RNA and a semi-quantitative RT-PCR reaction was performed

for detection of DR5 transcripts. The amplification of GAPDH was performed as a positive control. The data show that DETANONOate upregulates DR5 mRNA levels. Bottom panel: Upregulation of DR5 protein by western. PC-3 cells were treated or left untreated with 1000 μM of DETANONOate for 18 hr. Total cellular protein was extracted and separated by SDS-PAGE and transferred onto nitrocellulose membrane as described in methods. The membrane was stained with anti-DR5 mAb. Levels of β-actin were used to normalize the protein expression. The blots represent one of three separate experiments. Densitometric analysis was performed. The data show that DETANONOate upregulates DR5 protein expression.

Figure 3. DETANONOate inhibits NF-κB and YY1 DNA-binding activities and inhibits YY1 expression. Nuclear extracts from PC-3 cells were treated or left untreated with DETANONOate (500 or 1000 μM) and then were analyzed by EMSA to assess NF-κB DNA-binding activity (Figure 3A) or YY1 DNA-binding activity (Figure 3B Top panel). Relative NF-κB and YY1 DNA-binding activity was determined by densitometry analysis. YY1 protein expression was determined by using PC-3 cells treated with DETANONOate (500 or 1000 μM) for 18 hr. The membrane was stained with polyclonal anti-humanYY1 antibody. The blots represent one of two separate experiments (Figure 3B Bottom panel). The findings demonstrate that treatment of PC-3 cells with DETANONOate results in inhibition of NF-κB and YY1 DNA-binding activity and also inhibition of YY1 protein expression.

Figure 4. Specific inhibition of YY1 expression induces up-regulation of DR5 expression and sensitizes PC-3 cells to TRAIL-mediated apoptosis. The PC-3 cells were transfected using the SureSilencing TM siRNA for YY1 or siRNA negative control. (A) RT-PCR for YY1 was performed and the data show inhibition of YY1 transcription by siRNA YY1. (B) The surface expression of DR5 was determined by flow cytometry analysis as described in methods. The data represent the mean fluorescence intensity (MFI) and are the mean of three independent experiments. *$P < 0.05$, medium *vs* cells transfected with siRNA YY1. (C) After transfection, the cells were treated or left untreated with different concentrations of TRAIL (1 or 2.5 ng/ml) for 18 h. Fixed and permeabilized PC-3 cells were stained with FITC-labeled anti-active-caspase-3 and then analyzed by flow cytometry as described in methods. The data are the mean of three independent experiments. *$p < 0.05$. The findings reveal that YY1 negatively regulates DR5 expression and inhibition of YY1 sensitizes PC-3 cells to TRAIL-mediated apoptosis.

Figure 5. YY1 negatively regulates DR5 transcription. The *SacI-NcoI* fragment of the 5'-flanking region of the DR5 promoter (pDR5) was subcloned into the SacI-NcoI site of pGVB2 luciferase assay vector (Toyo ink, Tokyo, Japan). The pDR5 promoter with the 5'-deletion mutant –605 that includes the YY1 binding site (pDR5/-605) was generated with deletion kits (Takara, Tokyo, Japan) (Yoshida, et al, 2001). The pDR5 plasmids missing the YY1 binding sequence (pDR5-YY1 mutant) was generated by using the QuikChange site-directed mutagenesis method as described in Materials and Methods. (A) The PC-3 cells were transfected with 10 μg of pDR5, pDR5-YY1 mutant or pDR5/-605 by electroporation as described in methods. 36 h after transfection the cells were

harvested and the luciferase activity was determined. The data show that the PC-3 cells transfected with either the pDR5-YY1mutant or the pDR5/-605 show a significant increase of luciferase activity (3 fold). (B) PC-3 cells were transfected with 10 μg of pDR5, pDR5 YY1 mutant or pDR5/-605 by electroporation as described in methods. 24 hr after transfection the cells were treated or not treated with DHMEQ (2μg/ml) for 18 hr. The cells were harvested and luciferase activity was determined. The data show that the NF-κB inhibitor DHMEQ augmented luciferase activity in both constructs and suggests that NF-κB regulates DR5 in YY1. The data represent the % of control and are the mean of two independent experiments.

Figure 6. Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL. This figure schematically demonstrates that treatment of PC-3 cells with NF-κB or YY1 inhibitor and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by the inhibitor, which partially inhibits NF-κB and YY1 DNA-binding activity. Inhibition of YY1 transcription diminishes its repressor activity in the DR5 promoter, and this results in the upregulation of DR5 transcription. Signal II is provided by TRAIL and combination of inhibitors and TRAIL results in apoptosis and synergy.
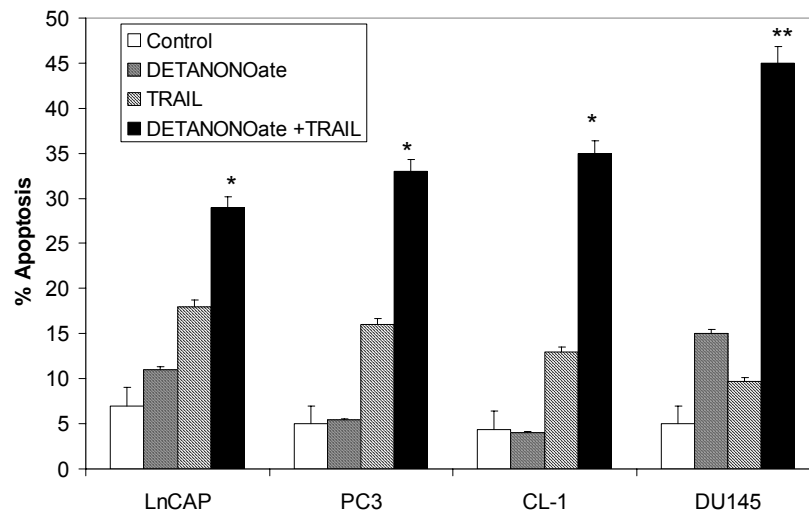
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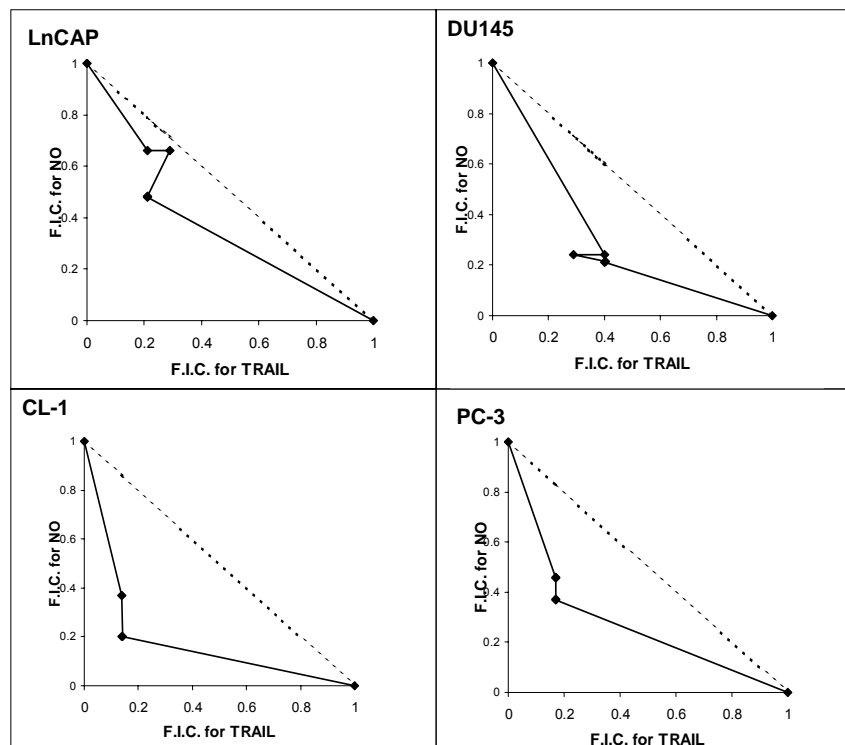
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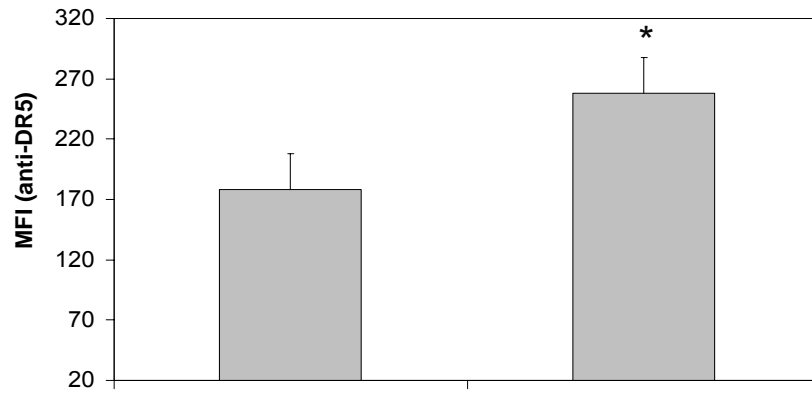
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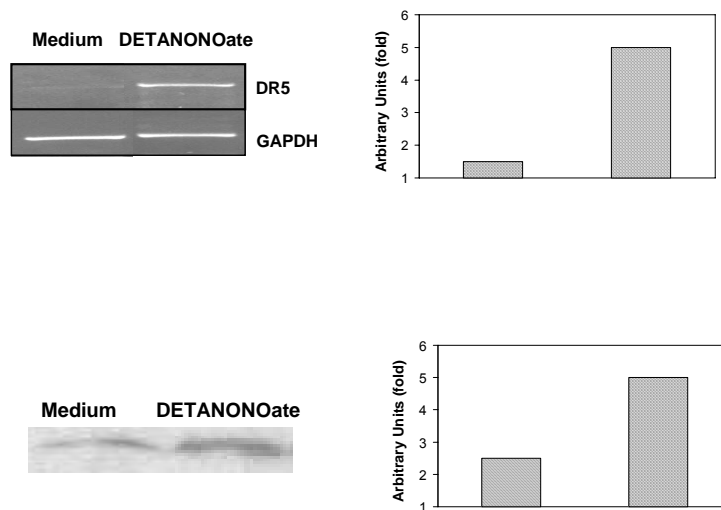
Figure 2(B)

Figure 3(A)

Figure 3(B)

siRNA YY1   siRNA Control

L   15   10   5   15   μl/ml

→ YY1

→ GAPDH

Figure 4(A)

B

C
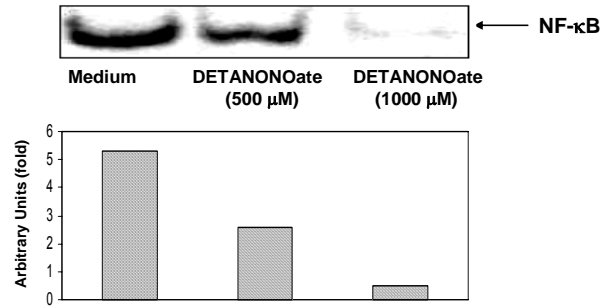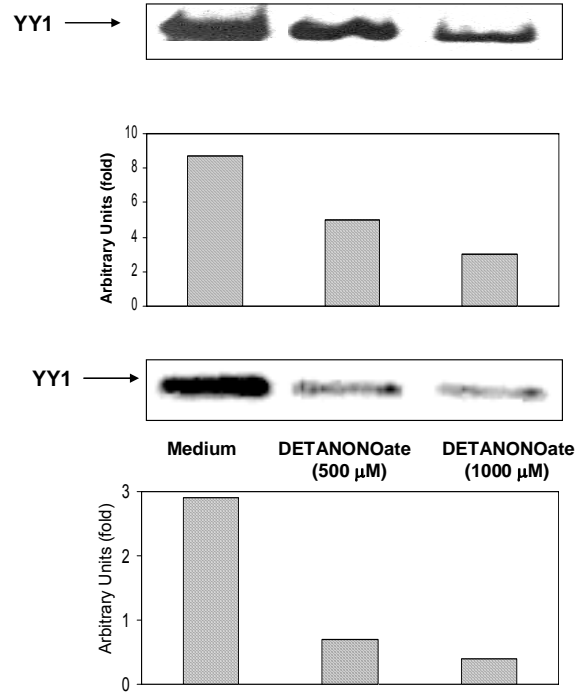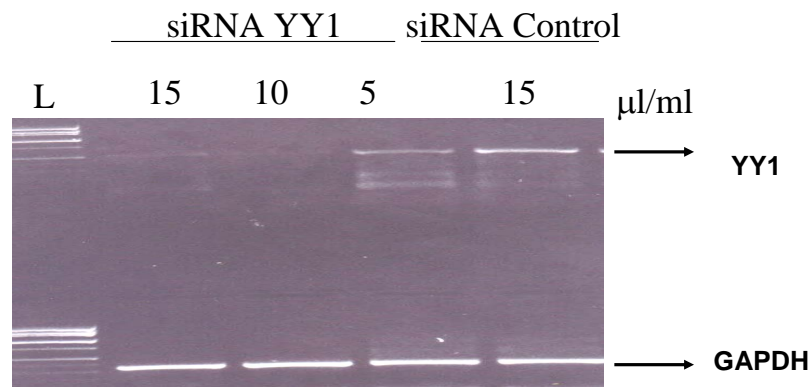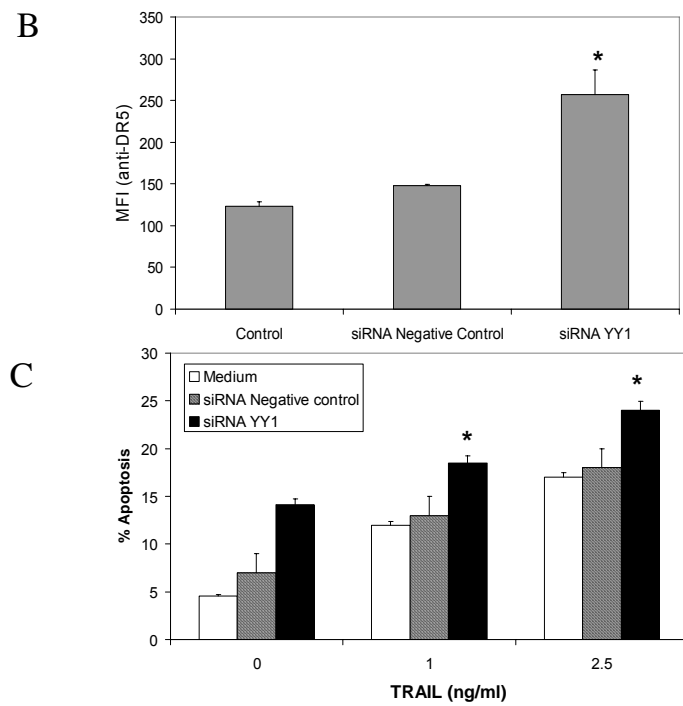
Figure 4

Figure 5(A)

Figure 5(B)

Sensitization to TRAIL apoptosis

Resistance to TRAIL apoptosis

DR5

DR5

NF-kB

YY1

DR5

DR5
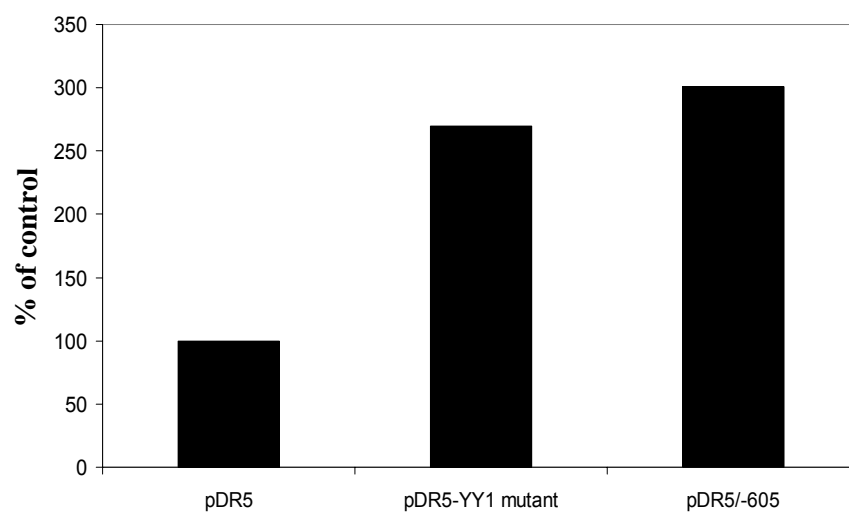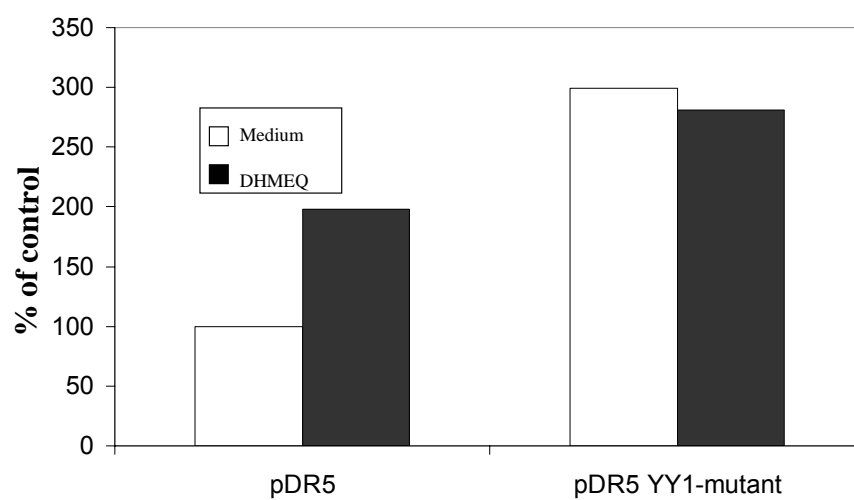
Figure 6
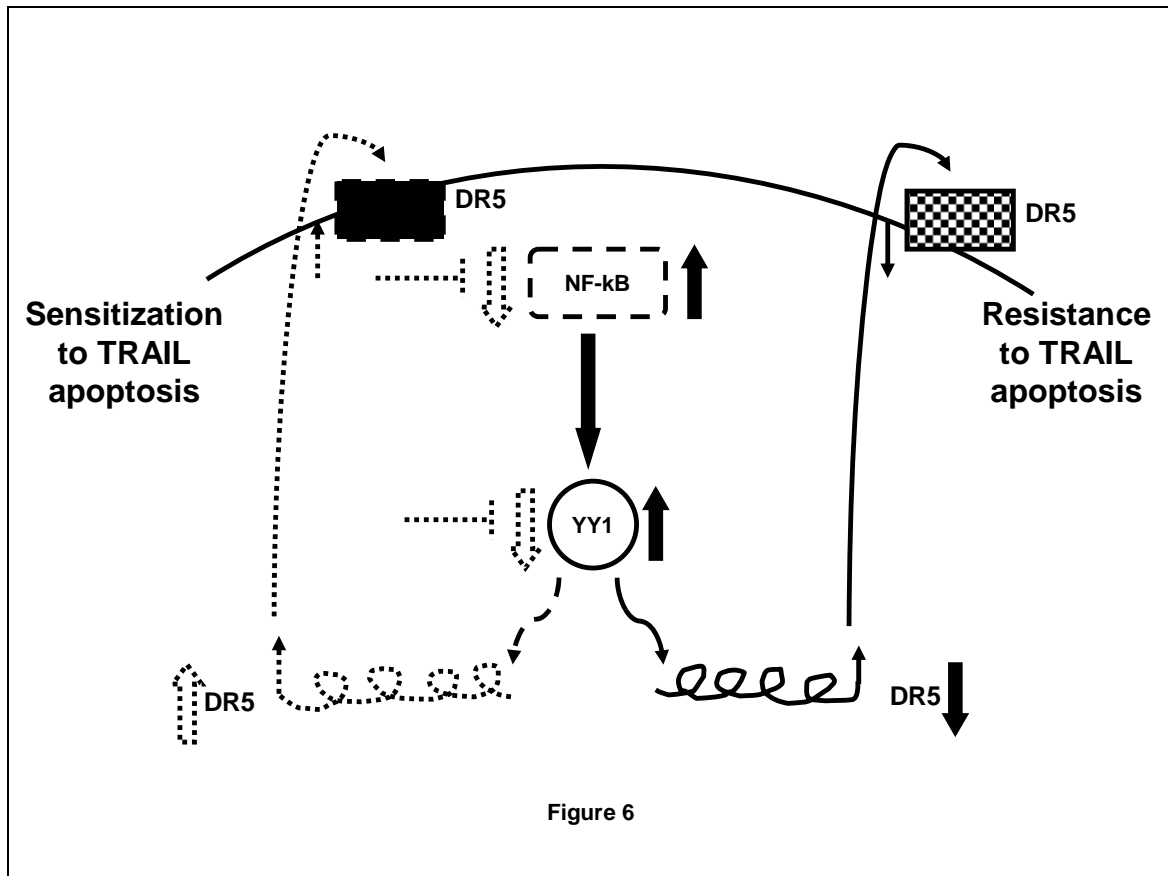
Involvement of the TNF-α autocrine/paracrine loop, via NF-κB and YY1, in the regulation of tumor cell resistance to Fas-induced apoptosis.

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Running title: TNF-α-mediated regulation of Fas resistance

Key words: Yin-Yang 1, NF-κB, TNF-α, prostate cancer, Fas-induced apoptosis

ABBREVIATIONS USED

NO: nitric oxide

sTNF-RI: soluble tumor necrosis factor receptor 1

TNF-α: tumor necrosis factor alpha

YY1: Yin-Yang 1

EMSA: Electrophoretic mobility-shift assay

siRNA: Small-interfering RNA

NF-κB: Nuclear factor kappa B

PE: Phycoerythrin

FITC: Fluorescein isothiocyanate

PAGE: Polyacrylamide gel electrophoresis

FBS: Fetal bovine serum

ABSTRACT

This study investigated the role of tumor-derived TNF-α autocrine/paracrine loop in the regulation of tumor-cell sensitivity to Fas-induced apoptosis. We have reported that Fas expression and sensitivity to FasL is negatively regulated by the transcription repressor factor Yin Yang 1 (YY1). We hypothesized that TNF-α-induced activation of NF-κB and YY1 may negatively regulate Fas expression and sensitivity to Fas-induced apoptosis. This hypothesis was tested in PC-3 prostate cancer cells which synthesize and secrete TNF-α and express constitutively active NF-κB and YY1. Treatment of PC-3 cells with TNF-α (10 units) resulted in increased NF-κB and YY1 DNA-binding activity, upregulation of YY1 expression, downregulation of surface and total Fas expression and induced-resistance of PC-3 to Fas agonist antibody CH-11-induced apoptosis. In contrast, blocking the binding of secreted TNF-α to TNF-RI with soluble sTNF-RI resulted in significant inhibition of both NF-κB and YY1 DNA-binding activity, downregulation of YY1 expression, upregulation of Fas expression and sensitization to CH-11-induced apoptosis. The regulation of YY1 expression and activity by NF-κB was demonstrated by the use of the NF-κB inhibitor Bay11-7085 and by using a GFP reporter system whereby deletion of the YY1 tandem binding site in the promoter significantly enhanced GFP expression. The direct role of YY1 in the regulation of PC-3 resistance to CH-11-induced apoptosis was shown in cells transfected with siRNA YY1 whereby such cells exhibited upregulation of Fas expression and were sensitized to CH-11-induced apoptosis. These findings demonstrate that the TNF-α autocrine-paracrine loop is involved in the constitutive activation of NF-κB and YY1 in the tumor cells leading to inhibition of Fas

expression and resistance to Fas-induced apoptosis. These findings also reveal new targets such as TNF-α, NF-κB and YY1 whose inhibition can reverse tumor cell resistance to Fas-mediated apoptosis.

INTRODUCTION

Cytotoxic lymphocytes kill target cells by various mechanisms including perforin/granzymes and the TNF-α superfamily that kills primarily by apoptosis (1). Tumors that develop anti-apoptotic mechanisms to resist chemotherapeutic drugs/radiation-induced apoptosis can also develop cross-resistance to immune cytotoxic lymphocytes (2, 3). The molecular mechanisms that govern anti-apoptotic resistance in cancer cells are numerous and vary from one type of tumor to another. Our recent findings revealed a novel mechanism that underlines tumor cell resistance to immune-mediated apoptosis. We have reported that resistance to Fas-mediated apoptosis of human ovarian and prostate cancer cell lines is due, in part, to the repressor activity of the transcription factor Yin Yang 1 (YY1) (4). We showed that YY1 negatively regulates Fas expression and sensitivity to Fas-mediated apoptosis; hence, inhibition of YY1 DNA-binding activity resulted in up-regulation of Fas expression and sensitization of tumor cells to Fas-mediated apoptosis. Changes in Fas expression and activity have been reported in many types of tumors (5-7).

YY1 is a 414-amino acid KRUPPEL-related zinc finger transcription factor that binds to the CG (A/C) CATNTT consensus DNA element located in promoters and enhancers of many cellular and viral genes (8-10). YY1 is a transcription factor that can act as a transcriptional repressor, activator, or initiator element binding protein (9, 11). The transcription activity of YY1 can be regulated by viral onco-proteins such as adeno-virus E1A (12). The transcription factor YY1 has been identified as a potential repressor factor for several genes such as the human interferon-γ gene (13, 14), the GMCSF

promoter (15, 16) and the IL-3 gene promoter (17). YY1 also regulates p-53-dependent transcription (18).

The transcription factor NF-κB is an important regulator of cells' ability to undergo apoptosis. NF-κB coordinates the expression of many genes involved in the regulation of inflammation, immune response, cell proliferation and apoptosis. In its anti-apoptotic capacity, NF-κB attenuates TNF-α–induced apoptosis through upregulation of anti-apoptotic gene products (19, 20). Positive regulation of Fas transcription has been shown to depend on NF-κB (21, 22). However, negative regulation of Fas expression may also take place indirectly via a transcription repressor such as YY1 (4).

Computer-based transcription search (TESS) analyses of the promoter region of the YY1 gene revealed the presence of 4 NF-κB putative binding sites clustered within the promoter of YY1 (-227bp from transcription site). Tumor cells, in general, exhibit constitutively active NF-κB which might regulate YY1 expression and activity. The constitutive activation of NF-κB in some tumors may be due to autocrine-paracrine loops of tumor-derived factors such as TNF-α, IL1-β, IL-6 (23). Accordingly, we hypothesized that one mechanism of tumor cell resistance to Fas may result from the activation of YY1 by NF-κB and consequently YY1 may negatively regulate Fas expression and sensitivity of tumor cells to Fas-induced apoptosis. This study tested this hypothesis. We have chosen the prostate cancer cell line PC-3 as a model system since it has been reported that PC-3 cells secrete TNF-α and express constitutively activated NF-κB (24). The following questions were addressed: 1) Does TNF-α secreted by PC-3 cells participate in the constitutive activation of NF–κB and YY1 via an autocrine/paracrine loop? 2) Does activation of NF-kB and YY1 negatively regulate Fas expression and sensitivity to Fas-

induced apoptosis? and 3) Does YY1 directly regulate Fas expression and resistance to Fas-induced apoptosis?

MATERIALS AND METHODS

Cell Culture and Reagents

The human androgen-independent PC-3 cell line is a metastatic bone-derived prostatic adenocarcinoma. PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PC-3 cells express low surface Fas and are resistant to Fas ligand-induced apoptosis. SW480 and SW620 cell lines were derived from a colon carcinoma of the same individual with the latter being from an advanced-stage, metastatic tumor (25, 26). K562 is known to be Fas-resistant whereas Raji is a Fas-sensitive lymphoma line (27).

The cell cultures were maintained as monolayers on plastic petri dishes. All the cells were maintained at 37ºC and 5% carbon dioxide in RPMI 1640 (Life Technologies Bethesda, MD), supplemented with 10% heat-inactivated FBS, 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) $_L$-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Life Technologies). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to experimental treatment.

The human recombinant TNF-α and human recombinant sTNF-RI were obtained from PeproTech, Inc (Rocky Hills, NJ). The cytotoxic anti-Fas monoclonal antibody (IgM, clone CH-11) and the Fas surface-staining monoclonal antibody (IgG$_1$, clone UB2) were purchased from Biomedical Co. (Thousand Oaks, CA). The rabbit anti-YY1 polyclonal antibody was obtained from Geneka (Montreal, Canada). FITC-conjugated anti-active caspase-3 and FITC-conjugated IgG were purchased from PharMingen (San

Diego, CA). The specific NF-κB inhibitor Bay 11-7805, a specific inhibitor of IκBα phosphorylation (28) was obtained from Calbiochem (San Francisco, CA).

Cytokine Treatment

Log phase PC-3 cells were used to seed six-well plates at approximately $5X10^5$ cells/ml and the cells were grown in 2 ml of medium as described above in 10% FBS for 24 h to approximately 70% confluence. The cells were synchronized and treated with 1% FBS for 18 h prior to treatment with TNF-α (10 U/ml) in serum-free RPMI medium for 24 h. Untreated cultured PC-3 cells in serum-free RPMI medium were used as a control for basal expression levels in the absence of exogenous cytokine.

Reporter system and site directed mutagenesis

The human Ornithine Decarboxylase Antizyme 1 (ODA1) minimal promoter (29) containing 201 bp upstream of the translation initiation site that includes an unique wild type responsive site (cgccattttgcga) for the transcription repressor Yin Yang 1 (YY1) was amplified by PCR using the forward primer 5'-CGG GCG CGA CTT TTT TTC CCG GC-3' and the reverse primer 5'-CCG GCC GCT GGG GTC CGA AAC CAG-3'. Genomic DNA extracted from cultured PC3 cells was used as template. PCR amplifications were conducted using the Advantage-HF2 system (Clonetech, Palo Alto, CA) following the manufacturer's recommendations. The gel-purified amplicon was ligated to the green fluorescent protein (GFP)-based pGlow-TOPO® reporter vector (Invitrogen, Carlsbad, CA) and further screened and sequenced in order to confirm fidelity and orientation of the construct (pGlow-OAZmp/WT-YY1). We further

generated one more construct whereby the YY1 cis-acting element (cg**ttg**ttttgcga) was mutated using the GeneTailor^TM Site-Directed Mutagenesis System (Invitrogen) following the manufacturer's recommendations. We confirmed the mutated reporter construct (pGlow-OAZmp/Mu-YY1) by automated sequencing. GFP-based reporter activity from transfected cells with these constructs was analyzed by direct fluorescence emission at 510 nm using excitation at 395 nm in a Fluorometer (Perkin Elmer Applied Biosystems, Foster City, CA).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For each of the cell lines, total RNA was extracted and purified from $\sim1X10^6$ cells for each experimental condition by a single-step monophasic solution of phenol and guanidine isothiocyanate-chloroform using Trizol® reagent (Life Technologies, Inc.). Three μg of total RNA was reverse-transcribed to first strand cDNA for 1 h at 42°C with SuperScript^TM II reverse transcriptase (Life Technologies, Inc) in a 20 μL reaction and performed per the manufacturer's specifications using random primers. Amplification of 1/10 of these cDNA by PCR was performed using the following gene-specific primers: YY1 (forward) (5'-GAA AAC ATC TGC ACA CCC ACG GTC C-3'), YY1 (reverse)(5'-GTC CTC CTG TTG GGA CCA CAC-3'), and Fas (forward)(5'-ATG CTG GGC ATC TGG ACC CT-3'). Internal control for equal cDNA loading in each reaction was assessed using the following gene specific glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers: GAPDH (forward)(5'-GAA CAT CAT CCC TGC CTC TAC TG-3') and GAPDH (reverse)(5'-GTT GCT GTA GCC AAA TTC GTT G-3'). PCR amplification of each DNA sequence was carried out by the "Hot Start" method

using Titanium Taq™ polymerase (Clontech) with the following one-step thermal cycling

incubation: 95°C/30 s, 68°C/1 min for 30 (Fas and YY1) or 25 (GAPDH) cycles, with a

final extension at 68°C/3 min. The number of cycles was established based on

preliminary titration of the relative amount of amplified product for each gene

representing the linear phase of the amplification process. The amplified products were

resolved on 1.5% agarose gel electrophoresis and their relative concentrations were

assessed by densitometric analysis of digitized ethidium bromide-stained image,

performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the

public domain NIH Image J Program (available on the internet).

Western Blot Analysis

PC-3 cells were cultured at a low serum concentration (0.1%) 18 h prior to each

treatment. After incubation, the cells were maintained in serum-free medium (control), or

treated with TNF-α (1, 10, and 100 U/ml-24 h). The cells were then lysed at 4°C in

RIPA buffer {50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate,

150mM NaCl} and supplemented with one tablet of protease inhibitor cocktail, Complete

Mini Roche (Indianapolis, IN). Protein concentration was determined by a DC protein

assay kit Bio-Rad (Hercules, CA). An aliquot of total protein lysate was diluted in an

equal volume of 2XSDS sample buffer 6.2mM Tris (pH6.8), 2.3% SDS, 5%

mecraptoethanol, 10% glycerol, and 0.02% bromphenol blue and boiled for 10 minutes.

The cell lysates (40μg) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad)

and were subjected to Western blot analysis as previously reported (30). Levels of β-actin

were used to normalize the YY1 expression. Relative concentrations were assessed by

densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

Nuclear Extracts Preparation

Nuclear extract preparations were done as previously described (4). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbeco PBS (Cellgro). After washing, cells were lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40) on ice for 5 min. Samples were centrifuged at 300 g at 4^oC for 5 min. The pellet was washed twice in NP40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated 10 s at 4^oC. The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at $–80^o$ C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche.

EMSA

Nuclear proteins (5μg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB and YY1 using EMSA Kit Panomics^{TM} (Panomics, Inc. Redwood City, CA) following the manufacturer's instructions. 10 μl of the reaction was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot® SD semi-dry Transfer

cell System (Bio-Rad, Hercules, CA)). The blotted membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide consensus sequences for NF-κB are as described: 5'-AGTTGAGGGGACTT TCCCAGGC-3' for YY1: 5'-CGCTCCGCGGCCATCTTGGCGGCTGGT-3'. Relative concentrations were assessed by densitometric analysis as mentioned above.

Caspase-3 Activity

PC-3 cells were grown in a six-well plate at a low serum concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in serum-free medium (control), or treated with TNF-α (10 U/ml-24 h), CH-11 antibody (30 ng/ml-12 h) or a combination of TNF-α and CH-11 antibody. Some samples were treated and some were left untreated with recombinant sTNF-RI (0.3 μg/ml). At the end of the incubation period, the cells were washed once with ice cold 1XPBS and were resuspended in 200ul of the cytofix/cytoperm solution (PharMigen, San Diego, CA) for 20 min. Thereafter, the samples were washed twice with ice cold 1Xperm/wash buffer solution (PharMigen) and were stained with FITC-labeled anti-active-caspase-3 mAb for 30 min (light protected). The samples were subsequently washed once with 1Xperm/wash buffer solution and 200 μl of 1XPBS was added prior to flow cytometry analysis (Coulter). As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

siRNA Transfections

PC-3 cells were cultured in 1 ml of RPMI medium supplemented with 5% FBS. Transfections were performed by using lipofectamine 2000 CD Reagent supplied by Invitrogen (Life Technologies, Carlsbad, CA, USA) and the SureSilencing™ siRNA kit supplied by SuperArray Bioscience Corporation (Fredrick, MD) according to the manufacturer's instructions. Briefly, 3 μl of YY1 siRNA or a negative control of siRNA solution were incubated with 4 μl of the transfection reagent in serum-free RPMI medium 1640 for 25 min to facilitate complex formation. The resulting mixture was added to PC-3 cells cultured in a 24-well plate with 1 ml of medium. To determine the extracellular expression of Fas, the cells were harvested 36 h after transfection and stained with anti-Fas monoclonal antibody for 30 min followed by anti-mouse IgG-PE for 20 min. The expression was then analyzed by flow cytometry. To determine PC-3 sensitization to Fas-mediated apoptosis, 24 h after transfection the cells were treated for 18 h with CH-11 (5 and 10 ng/ml) and fixed and permeabilized for anti-active caspase-3-FITC antibody staining. The cells were then analyzed by flow cytometry under the same conditions described above. To determine the inhibition of YY1 transcription by YY1 siRNA, specific RT-PCR for YY1 was performed (data not shown).

Statistical Analysis.

The experimental values were expressed as the mean ± SEM for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Students' *t* test were used

for comparison between two groups. Significant differences were considered for probabilities $< 5\%$ ($p < 0.05$).

RESULTS

Endogenously secreted TNF-α regulates YY1 gene expression and negatively regulates Fas gene expression

We have previously reported that the transcription repressor YY1 negatively regulates Fas transcription (4). We examined whether tumor-derived TNF-α is involved in the regulation of YY1 and Fas expression. We first examined the effect of exogenous TNF-α on YY1 expression. PC-3 cells cultured in 10% FBS or serum-free were treated with TNF-α and YY1 expression was measured by flow cytometry and by western. Base-level of YY1 expression, as determined by flow, decreased when PC-3 cells were cultured in the absence of serum compared to cells cultured in 10% FBS . Serum-free PC-3 cells cultured in the presence of TNF-α (10 U/ml) showed a significant upregulation of YY1 expression compared to serum-free untreated PC-3 cells (Figure 1A). The TNF-α-induced upregulation of YY1 expression examined by flow was confirmed by western blot analysis, whereby, TNF-α treatment of PC-3 cells significantly upregulated the expression of YY1 in a concentration-dependent manner (Figure 1B). Since YY1 expression negatively regulates Fas expression (4) and TNF-α upregulated YY1 expression, we expected that TNF-α treatment of PC-3 cells will inhibit Fas expression. Accordingly, PC-3 cells were cultured for 24 h in the presence or absence of TNF-α (10U/ml) under serum-free conditions and surface Fas expression was monitored. Surface

Fas expression was significantly decreased in PC-3 cells treated with TNF-α compared to untreated cells (Figure 1C). These findings demonstrated that there was a correlation between TNF-α-induced upregulation of YY1 expression and downregulation of Fas expression.

Based on the expected findings obtained above, following treatment of PC-3 cells with exogenous TNF-α, we examined whether tumor-derived TNF-α mimics exogenous TNF-α via an autocrine/paracrine mechanism. Hence, we predicted that interruption of the TNF-α loop may inhibit the constitutive expression of YY1 and thus, may result in upregulation of Fas expression. Blocking of the TNF-α autocrine/paracrine loop was done by the use of recombinant sTNF-RI, which should inhibit TNF-α-TNF-RI interaction and cell activation. Treatment of PC-3 cells with different concentrations (0.1 and 0.3 ug/ml) of recombinant sTNF-RI resulted in a significant decrease of YY1 expression when compared to untreated cells, and the inhibition of YY1 expression was a function of the sTNF-RI concentration used (Figure 1D). These findings demonstrate that PC-3-derived TNF-α, acting via an autocrine/paracrine loop, is involved in the constitutive regulation of YY1 expression and, consequently, downregulation of Fas transcription and expression.

Regulation of both YY1 expression and DNA-binding activity by TNF-α via activation of NF-κB

It is well-known that TNF-α activates NF-κB (31, 32) and we have shown above that TNF-α regulates YY1 and Fas expression; thus, we examined whether TNF-α−mediated activation of NF-κB was involved in the regulation of both YY1 and Fas

expression. The YY1 core promoter contains a significant cluster of NF-κB responsive elements (Figure 2A). We postulated that if NF-κB was involved in the regulation of YY1 and Fas, that inhibition of NF-κB activity would correlate with inhibition of YY1 expression and enhancement of Fas expression. This hypothesis was tested using a specific NF-κB inhibitor, Bay11-7085 (28) or inhibition of TNF-α-induced constitutive NF-κB activity by sTNF-R1. PC-3 cells were treated with different non-toxic concentrations of the NF-κB inhibitor Bay 11-7085 (0, 1, 2 or 3 μM/ml) for 1 h and the cells were then cultured in the presence or absence of TNF-α (10 U/ml) in serum-free conditions and YY1 expression was examined. YY1 expression in PC-3 cells was significantly inhibited by Bay 11-7085 and this inhibition was a result of the Bay 11-7085 concentration used. Further, the observed TNF-α-mediated upregulation of YY1 expression was also significantly inhibited by the NF-κB inhibitor Bay 11-7085 in a concentration-dependent manner (Figure 2B). These findings demonstrate that there was a correlation between inhibition of NF-κB and inhibition of YY1 expression.

Since the above findings demonstrated that both TNF-α and NF-κB regulate YY1 expression, we examined the effect of TNF-α treatment on the DNA-binding activity of NF-κB and YY1 in PC-3 cells by EMSA. Nuclear extracts from PC-3 cells grown in serum-free medium and treated with TNF-α (10 U/ml) for 24 h showed augmented NF-κB (Figure 3A Top panel) and YY1 (Figure 3A Bottom panel) (lane 3)-DNA-binding activity compared to both untreated serum-free (lane 2) and serum-containing controls (lane 1). The involvement of NF-κB on the regulation of YY1 DNA-binding reaction was corroborated by competition assays performed with a 10-fold excess of unlabeled NF-κB

and YY1 oligonucleotides, respectively (data not shown). The specific role of NF-κB in the regulation of YY1 DNA-binding activity was corroborated by the use of the NF-κB inhibitor Bay 11-7085. Treatment of PC-3 cells with various concentrations (0.5, 1, 2, 3 μM) of Bay 11-7085 inhibited both NF-κB (Figure 3B Top panel) and YY1 (Figure 3B Bottom panel) DNA-binding activity and the inhibition was a function of the inhibitor concentration used. These findings demonstrate that there was a good correlation between the inhibition of NF-κB activity and the inhibition of YY1 DNA-binding activity.

Based on the above findings on the role of NF-κB in the regulation of both YY1 expression and DNA-binding activity, we expected that the inhibition of the TNF-α autocrine/paracrine loop would mimic the inhibition of NF-κB by the Bay-11-7085 inhibitor, and would result in the inhibition of both NF-κB and YY1 DNA-binding activities. Accordingly, PC-3 cells were treated with sTNF-RI (0.3 and 0.6 ug/ml) for 18 and nuclear lysates were prepared for EMSA. Treatment with sTNF-RI significantly inhibited both NF-κB (Figure 3C Top panel) and YY1 (Figure 3C Bottom panel) DNA-binding activity. Altogether, these findings strongly suggest that tumor-derived TNF-α, via an autocrine/paracrine loop, regulates NF-κB activity and, in turn, NF-κB regulates YY1 expression and activity.

To determine whether NF-κB is involved in the regulation of YY1 transcription, transient transfection assays were performed. PC-3 cells were transfected with either the reporter vector pGlow-OAZmp/WT-YY1 which contains the human Ornithine decarboxylase antizyme 1 minimal promoter that includes an unique wild type responsive site of YY1 or with pGlow-OAZmp/Mu-YY1 in which the responsive site of YY1 was mutated. Twenty-four hours after transfection, the cells were treated with sTNF-RI (1 and

2 μg/ml) or with the specific inhibitor of NF-κB, Bay11-7085 (3 μM) and reporter activity was recorded. The baseline activity of the transfectants with WT-YY1 (Figure 4 Lane 3) was minimal and transfectants with the Mu-YY1 (lane 6) showed significant activity, suggesting that YY1 negatively regulates ODA1 activity. Hence, inhibition of YY1 by blocking the TNF-α loop by sTNF-R1 should inhibit YY1 and enhance ODA1 activity. Indeed, treatment of the cells with either sTNF-RI (Figure 4A) or the NF-κB inhibitor Bay11-7085 (Figure 4B) induced significant augmentation of GFP activity in the WT-YY1 transfectants. However, treatment of the Mu-YY1 with sTNF-RI did not alter the activity. In contrast, treatment of the Mu-YY1 with Bay 11-7085 induced significant inhibition of ODA1 activity, suggesting that the minimal promoter of the ODA1 contains other responsive sites beside YY1 that can explain the effect of NF-κB inhibition. These findings support the role of NF-κB in the regulation of YY1 transcriptional and repressor activities.

TNF-α-Dependent Activation of NF-κB Protects Human Cancer Cells Against Fas-Mediated Apoptosis via upregulation of YY1 activity

The above findings demonstrated that TNF-α upregulates YY1 expression and DNA-binding activity and, consequently, negatively regulates Fas expression. Thus, we expected that TNF-α would also confer resistance to CH-11-mediated apoptosis in PC-3 cells. Treatment of PC-3 cells cultured in the presence of 10% FCS with the FasL agonist antibody CH-11 resulted in moderate apoptosis (data not shown). In contrast, PC-3 cells grown under serum-free conditions and then treated with CH-11 antibody for 12 resulted in significant apoptosis. However, the addition of TNF-α to CH-11-treated PC-3 cells significantly inhibited apoptosis. This inhibition was overcome by the presence of sTNF-

RI (Figure 5A). These findings suggested that TNF-α-induced NF-κB activation was responsible in part for the resistance of PC-3 cells to CH-11-induced apoptosis. This was confirmed by the use of the NF-κB inhibitor, Bay11-7085. Treatment of PC-3 cells with CH-11 in the presence of Bay11-7085 significantly sensitized the cells to CH-11 induced apoptosis (Figure 5B). The sensitization achieved with Bay11-7085 was much greater than that achieve with sTNF-R1 since NF-κB activity was blocked pronouncely by Bay11-7085 than by blocking with sTNF-RI (see Figure 3).

The data above revealed that TNF-α regulates the resistance of PC-3 cells to Fas-mediated apoptosis through the activation of NF-κB. We have also shown that NF-κB regulates the activation of the transcription repressor YY1 and, in turn, YY1 negatively regulates Fas expression and sensitivity to CH-11-induced apoptosis. We performed experiments to directly demonstrate the role of YY1 in the regulation of Fas using cells transfected with siRNA YY1. Transfection of PC-3 cells with siRNA-YY1 resulted in significant upregulation of surface Fas expression as compared to cells transfected with siRNA negative control or to non-transfected cells (Figure 6A). Further, the siRNA-YY1 transfected cells showed significant potentiation of CH-11 induced apoptosis compared to controls (Figure 6B). These findings directly implicate the role of YY1 in the regulation of Fas expression and sensitivity of CH-11-induced apoptosis.

Fas/YY1 gene expression correlates with tumor cell sensitivity to Fas-mediated apoptosis

The above finding with PC-3 cells established the inverse relationship between YY1 expression and sensitivity to CH-11-induced apoptosis. This relationship was examined in other tumor cell lines. Semi-quantitative RT-PCR for the transcription

profile of YY1 and for Fas in five human tumor cell lines exhibited a wide range of sensitivity to Fas-mediated apoptosis. The cell lines were arranged in decreasing order of their sensitivity to Fas such that Raji and SW480 cells being the most sensitive, followed by PC-3 and SW620 cells which were moderately resistant, and K562 cells which were not sensitive to Fas-mediated apoptosis. The Fas/YY1 transcription ratios were used to assess whether the level of YY1 expression correlated with Fas resistance. An inverse correlation was found between YY1 and Fas sensitivity in the tested cell lines (Figure 6C). The Fas sensitive Raji and SW480 cell lines exhibited a Fas/YY1 ratio greater than one, while the Fas-resistant SW620, PC-3 and K562 cell lines exhibited Fas/YY1 ratios less than one. These findings suggested that the Fas/YY1 ratios appear to predict sensitivity to Fas-mediated apoptosis.

DISCUSSION

Evidence is presented which demonstrates that the autocrine-paracrine loop mediated by TNF-α in PC-3 cells regulates tumor cell expression of Fas and resistance to Fas-induced apoptosis. Endogenously secreted TNF-α regulates, in large part, the constitutively activated NF-κB in PC-3 cells. The role of NF-κB in the negative regulation of Fas expression and resistance to Fas apoptosis was found to correlate with NF-κB-induced regulation of the transcription repressor YY1. Both endogenous and exogenous TNF-α, via NF-kB activation, resulted in upregulation of both the expression and DNA-binding activity of YY1 and concomitant downregulation of Fas expression. The role of NF-κB in the regulation of YY1 repressor activity was corroborated using a luciferase reporter system and by the use of the NF-κB inhibitor Bay 11-7085. Several lines of evidence support the direct role of YY1, via NF-κB activation by tumor-derived

TNF-α autocrine-paracrine loop, in the negative regulation of Fas expression and resistance to Fas-induced apoptosis. Treatment of PC-3 cells with TNF-α upregulated YY1 expression and activity and downregulated Fas expression. In contrast, inhibition of TNF-α-mediated signaling resulted in upregulation of Fas expression and sensitization to CH-11-induced apoptosis. These findings were corroborated with tumor cells treated with inhibitors of NF-κB. Further, treatment of PC-3 cells with YY1 siRNA resulted in upregulation of Fas expression and sensitization to CH-11-induced apoptosis. Altogether, the findings of this study provide for the first time evidence for the role of tumor-derived TNF-α, via an autocrine-paracrine loop, in the downregulation of Fas expression and resistance to Fas-induced apoptosis by activation of the transcription factors NF-κB and YY1.

Several reports have demonstrated that tumor cells sensitize and secrete various cytokines and growth factors that play important roles in cell survival and cell growth via autocrine/paracrine loops (33). Likewise, such factors derived by non-tumor cells are also encountered by the tumor cells *in vivo* in their microenvironment (34, 35). It has also been reported that cytokines secreted by the tumor cells can regulate the sensitivity and resistance of tumor cells to various cytotoxic stimuli, partly due to stimulation of cell survival pathways and anti-apoptotic mechanisms and/or inhibition of pro-apoptotic-regulatory gene products (36). In this study, we have examined the role of tumor-derived TNF-α, via its effect by an autocrine-paracrine loop, for its involvement in the regulation of tumor cell resistance to Fas-induced apoptosis. The findings revealed that secreted TNF-α from the tumor cells was largely responsible for the constitutively activated NF-κB observed in PC-3 cells and upregulation of the expression and activity of YY1. The

constitutively activated YY1 was shown to negatively regulate Fas transcription and expression. This effect was due to YY1-induced repressor effect on the silencer region of the Fas promoter as previously described (4). TNF-α is a potent activator of NF-κB and NF-κB has been shown to regulate cell survival and numerous genes that are anti-apoptotic (23, 37). We show that inhibition of endogenous TNF-α secreted by PC-3 cells by sTNF-R1, thus neutralizing TNF-α–sTNF-RI-mediated signaling, significantly inhibited the constitutive NF-κB activity. These findings suggested that tumor-derived TNF-α is largely responsible for maintaining tumor cell survival with an anti-apoptotic phenotype.

The role of NF-κB in the negative regulation of Fas expression and resistance to CH-11-induced apoptosis in PC-3 cells was demonstrated by inhibiting NF-κB activity. This was performed by either neutralization of secreted TNF-α by sTNF-RI or by treatment with the specific NF-κB chemical inhibitor, Bay 11-7085. These treatments resulted in the upregulation of Fas expression and sensitization of PC-3 cells to CH-11-induced apoptosis. Related studies demonstrated that NF-κB regulates the survival of cells and also regulates the transcription of several anti-apoptotic gene products. Inhibition of NF-κB results in the sensitization of cells to various apoptotic stimuli (38). The present study provides a new insight, namely by YY1, that underlies the mechanism of NF-κB-induced regulation of tumor cell survival and resistance to Fas-induced apoptosis. In this study, TNF-α-mediated activation of NF-κB resulted in upregulation of YY1 expression and augmentation of YY1 DNA-binding activity. The endogenous YY1 expression and YY1 activity in PC-3 cells were shown to be regulated, in part, by constitutively activated NF-κB, and there was a good correlation between YY1

expression and YY1 DNA-binding activity. The role of NF-κB in the regulation of the repressor activity of YY1 was demonstrated in a reporter system whereby the YY1 binding sites were deleted from the promoter resulting in upregulation of luciferase activity. In addition, inhibition of NF-κB by Bay 11-7085 inhibited the repressor activity of YY1.

Based on our present findings demonstrating that TNF-α-induced NF-κB activation regulates in part YY1 expression and activity, we expected that Fas expression would be negatively regulated by both TNF-α and NF-κB. Indeed, treatment of PC-3 cells with TNF-α inhibited Fas expression and inhibited PC-3-mediated apoptosis by the Fas ligand agonist antibody CH-11. In contrast, blocking the TNF-α autocrine/paracrine loop-mediated activation of NF-κB by sTNF-RI resulted in upregulation of Fas and sensitization to CH-11-induced apoptosis. The direct role of YY1 in the regulation of Fas expression and sensitization to Fas was corroborated by transfection of PC-3 cells with YY1 siRNA . The transfected cells showed upregulation of Fas and sensitization to CH-11-induced apoptosis.

In contrast to our present findings, Ivanov *et. al.* (20) reported that p38 negatively regulates the expression of Fas via inhibition of NF-κB transcriptional activity in melanoma tumor cells. Inhibition of NF-κB activity correlated with significant downregulation of Fas expression and UV-induced apoptosis. The Fas promoter contains 3 NF-κB sites (39) and inhibition of p38 resulted in significant increase in Fas reporter luciferase activity. Our findings are not consistent with these findings, and the differences may reflect differences in the tumor system used and or the apoptotic stimulus. It is also possible that the counteracting regulation of Fas by NF-κB and YY1 may be balanced

based on levels of expression and activity. In the PC-3 system studied here, it is possible that YY1 activity is dominant over NF-κB-mediated effects whereas this may not be the case in the melanoma cell line studied.

A large number of genes has been found to be potentially regulated by YY1 and a large number of genes has been claimed to interact with YY1 (9). However, little is known about the transcriptional regulation of YY1. Patten *et. al.,* (40) reported that IL-1-β increases the abundance of YY1 in cardiac myocytes. Santiago *et. al.,* (41) demonstrated that YY1 is activated in rat vascular smooth muscle cells shortly after injury and this was due to endogenous FGF-2 mRNA, protein and DNA binding and transcriptional activity of YY1 that was increased 3-fold by FGF-2. Also FGF-1 has been shown to regulate YY1 expression in NIH 3T3 cells (42). The present findings demonstrate one mechanism of YY1 regulation, namely, the role of NF-κB or stimuli that activate NF-κB like TNF-α that result in the transcriptional regulation of YY1. Although YY1 is generally regarded as an ubiquitous protein expressed in many different tissues and cell types, YY1 is differentially regulated in different cell types. For example, expression of YY1 mRNA in NIH 3T3 cells has been shown to be affected by cell density and growth factors such as IFG-1 (42). Levels of YY1 activity also change during myoblast differentiation and during aging (43). We have found strong nuclear YY1 immunostaining in several cancer cell lines (AD10, SW620, SW480, and PC-3) (data not shown). Further, recent studies in our laboratory have demonstrated by immunostaining, using tissue arrays, overexpression of YY1 in prostate cancer (44). One mechanism of YY1 overexpression has been presented via the tumor-derived TNF-*a* autocrine-paracrine loop which activates both NF-κB and YY1. The present findings are schematically

diagrammed in Figure 7. Figure 7A schematically describes the effect mediated by TNF-α in PC-3 cells leading to cell resistance to Fas-induced apoptosis. Figure 7B shows various targets whose modifications reverse tumor cell resistance to immune-mediated stimuli. Overall, the findings suggest that overexpression of YY1 may be detrimental in the response of tumor cells to Fas-induced apoptosis and potentially to other immune-mediated stimuli.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Correlation between Fas expression and YY1 expression in tumor cell lines

A. YY1 and Fas expression in PC-3 cells

PC-3 cells were grown in RPMI 10% FSS, serum free medium alone or serum free medium with TNF-α (10 U/ml) as described in Materials and Methods. Fixed and permeabilized PC-3 cells were stained with anti-YY1 antibody and goat-anti-rabbit-PE and then analyzed by flow cytometry. The data are presented as mean fluorescence intensity and the mean of three independent experiments ***** $p < 0.05$, serum-free vs. cells treated with TNF-α.

B. TNF-α- dependent YY1 expression in PC-3 cells

PC-3 cells were grown in serum-free medium, untreated (lane 1) or treated for 24 h with 0.1, 1, and 10 U/ml of TNF-α (lanes 2, 3 and 4). Total cellular protein was extracted from the culture and tested for YY1 by western as described in methods. β-actin was also tested for loading. The relative YY1 expression was determined by densitometric analysis of the blots. The blots represent one of two separate experiments. $^{*}p < 0.05$, serum free vs. cells treated with TNF-α.

C. Surface Fas expression in PC-3 cells

PC-3 cells were treated as described above in (A). The cells were stained for surface expression using anti-Fas monoclonal antibody as described. The data are provided as mean fluorescence intensity (MFI) and the MFI represents the mean of three independent experiments *p<0.05 serum-free versus treated cells with TNF-α.

D. Endogenous TNF-α is involved in the regulation of YY1 expression in PC-3 cells

PC-3 cells were grown in serum-free medium and then treated or left untreated for 24 h with recombinant sTNF-RI (0.1, 0.3 μg/ml). Fixed and permeabilized PC-3 cells were stained with anti-YY1 antibody and analyzed by flow cytometry. The data are

the mean of two independent experiments ***** *p* < 0.05, ****** *p* < 0.01 serum free vs. cells

treated with sTNF-RI.

Figure 2. NF-κB mediates TNF-α dependent expression of YY1
A. NF-κB responsive elements in the YY1 core promoter
Sequence analysis of the YY1 proximal core promoter reveals the presence of four

putative cis-acting responsive elements for NF-κB.

B. NF-κB mediates TNF-α-dependent expression of YY1
PC-3 cells were treated with different concentrations of the NF-κB inhibitor Bay11-

7085 (0, 1, 2 or 3 μM) for 1 h. PC-3 cells were then treated or left untreated for

24 h with TNF-α (10 U/ml) in serum-free conditions. Fixed and permeabilized PC-3

cells were stained with anti-YY1 antibody and analyzed by flow cytometry. The data

are presented as mean fluorescence intensity of two independent experiments. ***** *p* <

0.05, ****** *p* < 0.01 presence of Bay11-7085 (2.0 and 3uM)) vs. absence of Bay11-7085.

Figure 3. Regulation of YY1 DNA-Binding activity by NF-kB
A. TNF-α augments NF-κB and YY1 DNA binding activities
Nuclear extracts from PC-3 cells grown in RPMI 10% FCS or serum-free medium

treated or left untreated with TNF-α (10U/ml) were analyzed using EMSA to assess

NF-κB (upper panel) and YY1 (bottom panel) DNA-binding activity. Relative NF-κB

and YY1 DNA-binding activity was determined by densitometric analysis.

B. The specific NF-κB inhibitor Bay11-7085 inhibits both NF-κB and YY1 DNA-binding activities
Nuclear extracts from PC-3 cells grown in serum-free medium treated or left

untreated with Bay 11-7085 (0.5, 1, 2 and 3 μM) were analyzed using EMSA to

assess NF-κB (top panel) and YY1 (bottom panel) DNA binding activity. Relative

NF-kB and YY1 DNA binding activity was determined by densitometric analysis.

C. sTNF-RI inhibits both NF-κB and YY1 DNA binding activities

Nuclear extracts from PC-3 cells grown in serum-free medium treated or left

untreated with recombinant sTNF-RI (0.3, 0.6 μg/ml) were analyzed using EMSA to

assess the specific NF-κB (top panel) and YY1 (bottom panel) DNA binding activity.

Relative NF-κB and YY1 DNA binding activity was determined by densitometric

analysis.

Figure 4. The suppressor activity of YY1 is modulated via the TNF-α/ NF-κB pathway

PC-3 cells were transfected with 20 μg of either the pGlow-OAZmp/WT-YY1   (100

bp fragment of the enzyme ODA1 promoter that includes one YY1-responsive site)

or   pGlow-OAZmp-Mu-YY1 (the 100 bp fragment of the enzyme ODA1 promotor

missing the YY1-responsive site).Twenty four h after transfection the cells were

treated with (A) sTNF-RI (1 or 2 μg/ml) or (B) with the specific NF-κB inhibitor

Bay11-7085 (3 μM). Samples were harvested 24 h after treatment and assessed for

GFP activity with a fluorometer. **p<0.03

Figure 5. TNF-α protects PC-3 sensitivity to CH-11-induced apoptosis via NF–κB activation

A.   PC-3 cells were cultured in serum-free medium and were left untreated or treated

with TNF-α (10 U/ml) in the presence or absence of recombinant sTNF-RI (0.3

μg/ml) for 12 h. PC-3 cells were then treated or left untreated with CH-11 antibody

(30 ng/ml) for 12 h. Fixed and permeabilized PC-3 cells were stained with anti-

active caspase-3-FITC antibody and analyzed by flow cytometry as described in

methods. The data are calculated as percentage of control cells cultured in serum-

free medium. *p<0.05 compared to cells treated with CH-11.

B. PC-3 cells were treated the same as aforementioned in (A) except that Bay 11-7085

(2uM) was used. *p=0.05 compared to cells treated with CH-11 alone.

Figure 6. Transfection of PC-3 cells with siRNA YY1 upregulates surface Fas expression and sensitizes cells to CH-11-induced apoptosis.

PC-3 cells were transfected using the SureSilencing TM siRNA for YY1 or siRNA negative control. A. Thirty six h after transfection, surface expression of Fas was determined by flow cytometry as described in methods. The data represent the mean fluorescence intensity (MFI) and are the mean of three independent experiments. *p< 0.05, medium *vs* cells transfected with siRNA YY1. B. After transfection, the cells were treated or left untreated with different concentrations of CH-11 (5 or 10 ng/ml) for 18 h. Fixed and permeabilized PC-3 cells were stained with FITC-labeled anti-active-caspase-3 and then analyzed by flow cytometry as described in methods. The data are the mean of three independent experiments. *p<0.05.

C. Fas/YY1 ratios of gene expression and sensitivity to Fas-mediated apoptosis in five human tumor cell lines
The cell lines were synchronized and then cultured in RPMI with 10% FBS as described in methods. Total RNA was extracted and RT-PCR was used to examine the basal levels of YY1 and Fas expression. All the samples were normalized against GADPH. The ratios of Fas/YY1 were calculated and are shown. Furthermore, the cell lines were tested for sensitivity to Fas-mediated apoptosis using the CH11 anti-Fas antibody.

Figure 7. Schematic diagram illustrating the mechanism by which TNF-α regulates YY1 expression and activity via NF-κB and the regulation by YY1 of Fas expression and sensitivity to Fas apoptosis.
A. Binding of TNF-α (endogenously by autocrine-paracrine loop or exogenously) to TNF-R1 activates NF-κB which in turn activates the expression of TNF-α and YY1 genes.   As a result, YY1 binds to the silencer region of the Fas promoter and blocks

Fas expression leading to downregulation of Fas expression and resistance of cells to Fas-mediated apoptosis.

B. The addition of sTNF-RI or Bay11-7085 to the cells inhibits constitutive NF-κB activity and as a result, YY1 expression is down-regulated and Fas expression is increased and the cells become sensitized to Fas-mediated apoptosis.
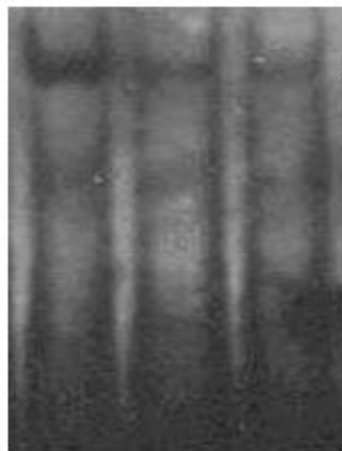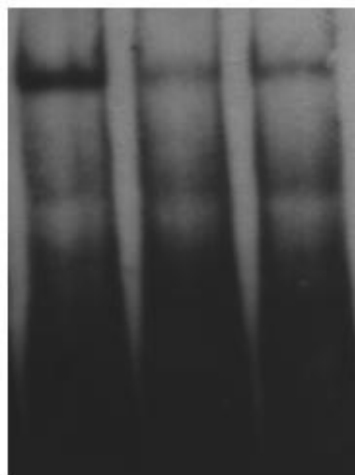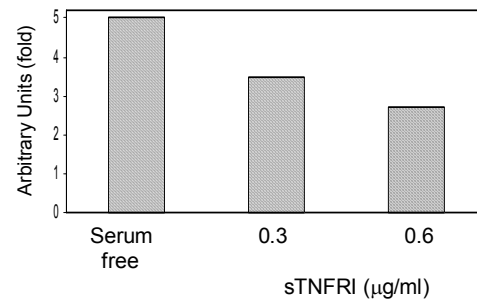
Figure 1

Figure 2

A

B

Figure 3

A

← NF-κB

← YY1

Figure 3

B

← NF-κB

← YY1

Figure 3

C

Figure 4

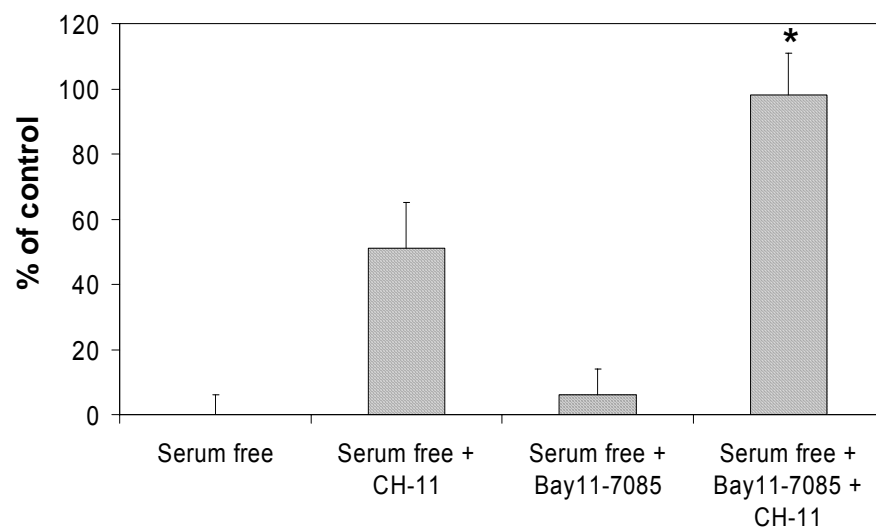Figure 5

Figure 6

A

B

C

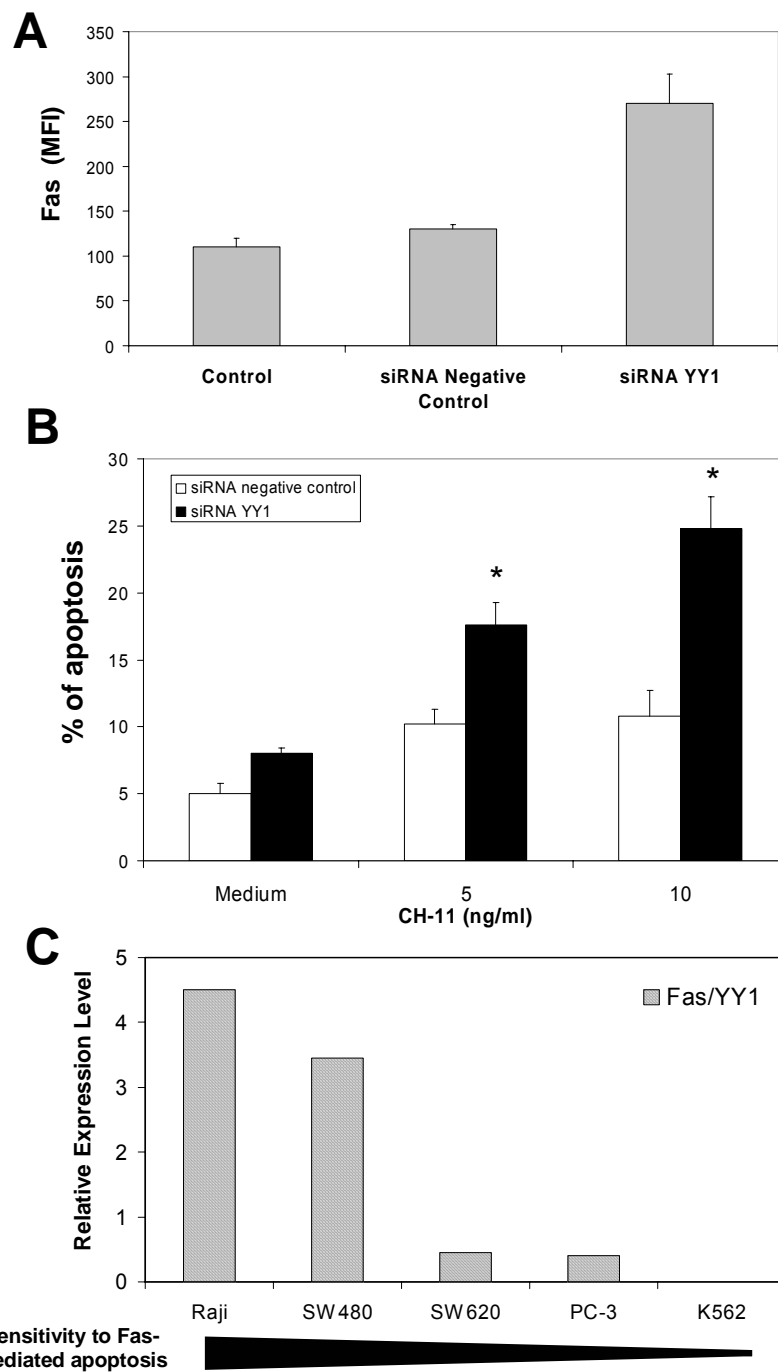Figure 7

January 12, 2006

Genotoxic agents sensitize prostate cancer cells to TRAIL-mediated apoptosis through inhibition of the transcription repressor YY1 and upregulation of DR5 expression

Running title: YY1 repression induces TRAIL-mediated apoptosis

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Keywords; Death receptor 5, Ying Yang 1; chemotherapeutic drugs, prostate tumors, apoptosis

ABSTRACT

Purpose: We and others have reported that several sensitizing agents (ex. VP-16, CDDP, ADR, chemical inhibitors, etc.) in combination with TRAIL result in reversal of resistance to TRAIL apoptosis. Sensitization correlated with the upregulation of DR5 expression. This study examined the mechanism underlying the upregulation of DR5 expression. We hypothesize that the sensitizing agents may inhibit a transcription repressor acting at the DR5 promoter.

Experimental Design: By examining the promoter of DR5, we detected the presence of one putative binding site for the transcription repressor YY1. We examined whether YY1 negatively regulates DR5 transcription and whether YY1 inhibition by the drug upregulates DR5 expression. We used PC-3 cells transfected with a luciferase reporter system (pDR5 WT) and plasmids in which the YY1 binding site was either deleted (pDR5 -605) and/or mutated (pDR5/YY1 mutant).

Results: Treatment of drug resistant PC-3 tumor cells with drugs (example CDDP, VP-16, ADR, vincristine) sensitized the tumor cells to TRAIL-induced apoptosis and apoptosis correlated with upregulation of DR5 expression and inhibition of YY1 expression and DNA binding activity. The findings revealed that the baseline reporter activity was significantly augmented in cells transfected with either the deleted or mutated plasmids. In addition, CDDP treatment augmented the luciferase activity in the W/T reporter system, whereas there was no augmentation in the deleted or mutant transfected cells. The direct role of YY1 in the upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis was further demonstrated in cells treated with siRNA YY1.

Conclusions: The findings demonstrate that drug-induced upregulation of DR5 and sensitization to TRAIL is mediated through inhibition of the transcription repressor YY1. Inhibition of YY1 correlated with sensitization to TRAIL-induced apoptosis.

INTRODUCTION

Chemotherapy and γ-radiation remain by far the widely used approaches for cancer control and treatment. The vast majority of the well-known potent antitumor chemotherapeutic agents display clinical activity against a wide variety of solid and hematogenous tumors. Most antineoplastic drugs are now thought to kill cells predominantly by triggering their apoptotic program. Their cytotoxic mode of action has been considered to be mediated through several different mechanisms, including interactions with DNA to form DNA adducts, primarily intrastrand crosslink adducts (such as in the case of alkylating platinum compounds) (1, 2), or complexes with DNA by intercalating between DNA base pairs, causing the helix to change shape such as in the case of anthracyclines. This simple act of changing the conformation of DNA can interfere with strand elongation by inhibiting DNA polymerase and can inhibit protein synthesis due to affects on RNA polymerase (3). These alterations are capable to activate several signal transduction pathways, including those involving ATR, p53, p73 and MAPK, and culminate in the activation of apoptosis (1). The mechanism of action of other classes of antitumor drugs like vinca alkaloids is more related to the inhibition of microtubule formation in the mitotic spindle resulting in an arrest of the dividing cells at metaphase (4).

DNA damage mediated apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of chemo-based tumor therapies. This may be explained by the fact that there is an array of alternate resistance mechanisms controlled by different families of genes, such as those involved in apoptosis. It has been observed that overexpression of Bcl-2 and Bcl-xL by constitutively active NF-κB renders tumor cells refractory to diverse therapeutic drugs, *in vitro* as well *in vivo* (5-8).

Alternative therapies have been considered including immunotherapy, both antibody and cell-mediated, with potential antitumor activity. Since CTLs mediate their killing by various mechanisms, including activation of death receptors in target cells, the idea of targeting death receptors and their respective signaling pathways to trigger apoptosis in tumor cells seems to be an attractive concept for cancer therapy. TRAIL/Apo-2 appears to be a relatively safe and most promising death ligand for clinical application among the other death ligands of the TNF-α family (TNF-α and Fas-Ligand) (9,10). It has been shown to exhibit potent tumoricidal activity against a variety of human cancer cell lines *in vitro* and *in vivo* in several xenograft tumor models with minimal, or no toxicity to nonmalignant human cells (10-12). TRAIL induces apoptosis in tumor cells by binding to death receptors TRAIL-R1/DR4

and TRAIL-R2/DR5 (13, 14). These receptors include an intracellular death domain which triggers the activation of the caspase signaling cascade after association of ligand with the receptor, with or without the involvement of mitochondria (15, 16)). Two more receptors for TRAIL have been identified to date, DcR1 and DcR2, however in contrast to DR5 and DR4 Dc receptors do not induce apoptosis due to the presence of mutations or deletions in the death domain (14, 17).

The majority of breast, prostate, ovarian and lung carcinoma, multiple myeloma and leukemia cells are resistant to apoptosis induced by TRAIL (18). Resistance of tumor cells to TRAIL appears to occur through the modulation of various molecular targets. These may include differential expression of death receptors, either low expression of DR4 and DR5, or increased surface levels of Dc receptors which can antagonize DR4 and DR5 for ligand binding, constitutively active Akt and NF-κB, overexpression of anti-apoptotic molecules such as c-FLIP and c-IAPs, mutations in apoptotic genes such as Bax and Bak, and defects in caspase signaling or caspase inhibition in resistant cells (18-20).

The above observations suggest that the use of TRAIL alone may not be a viable option to treat these tumors. To overcome this problem conventional chemotherapeutic and chemo preventive drugs and irradiation are already used as agents to enhance the therapeutic potential of TRAIL in TRAIL sensitive cells and to sensitize TRAIL-resistant cells to TRAIL-mediated apoptosis. Genotoxic drugs such as ADR, VP-16 and CDDP as well as γ-radiation have been shown to induce the expression of DR5 in a p53-dependent or p53–independent manner (9, 21-27). The last observation suggests that other transcription factors may also induce, or suppress death receptor expression, especially in those cells where DR5 expression seems to be p53–independent (28). The direct or indirect influence of many chemotherapeutic drugs on the activity of those factors is poorly studied, and its relative contribution in death receptors' expression profiles remains to be elucidated. By expressing more of the death receptor, TRAIL resistant cells may then become sensitive to TRAIL. TRAIL and drugs may also activate distinct apoptotic pathways, which may converge and further amplify the treatment response. However, the mechanisms by which most of the chemotherapeutic drugs sensitize tumor cells to TRAIL-mediated apoptosis still remain unknown.

Few studies focused on testing the regulation of sodium butyrate-induced DR5 expression in colon tumors have identified a functional Sp1 binding site that is responsible for regulation of DR5 expression (27, 29). We have also identified another binding site for the transcription factor Ying-Yang 1 (YY1) in the DR5 promoter region (-804 to -794 bp). YY1 is an ubiquitously expressed Zn finger

transcriptional regulator in numerous viral and cellular genes involved in the control of cell growth, development, differentiation and tumor suppression (30-33). Through interplay with various basal transcription factors (TATA binding protein, TAFs) (34, 35) and other transcriptional regulators (Sp1, c-Myc) (36, 37), as well as through binding to DNA response elements and to initiator sequences, YY1 can exert wide activities at target promoters acting either as activator, or repressor, or as initiator binding protein. In previous findings, we have reported that Fas expression is negatively regulated by the transcription repressor YY1 via binding of YY1 to the silencer region of the Fas promoter (38). Since there is also a binding site for YY1 in the DR5 promoter, we hypothesized that sensitizing by chemotherapeutic drugs may inhibit YY1 expression or DNA-binding activity and resulting in relieving the YY1-induced transcriptional repressor activity and subsequently upregulating DR5 transcription and expression.

The above hypothesis was tested by using as experimental model a human androgen-independent prostate cell line PC-3, which was sensitized by chemotherapeutic drugs (CDDP, VP-16, ADR and vincristine) to TRAIL-induced apoptosis. We addressed and examined the following: (1) Do the sensitizing drugs upregulate DR5 expression? (2) Do the sensitizing drugs inhibit YY1 expression/activity? (3) Does inhibition of YY1 upregulate DR5 expression and sensitizes the cells to TRAIL-induced apoptosis? (4) Does the DR5 promoter have a binding site for YY1 that negatively regulates DR5 transcription?

MATERIALS AND METHODS

Cell lines and Culture Conditions. The drug-resistant human androgen-independent prostate tumor cell line PC-3 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The prostate cells were cultured in RPMI 1640 (Cellgro, Mediatech, Inc, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, NY, USA), 50 I.U/ml penicillin and 50µg/ml streptomycin (both from Cellgro). Cell cultures were maintained at 37^0 C and 5% CO_2 for incubation.

Reagents. CDDP, VP-16 and ADR were purchased from Sigma (St. Louis, MO). Vincristine was obtained from Dr. Mizutani (Kioto, Japan). Stock solutions of ADR and vincristine were prepared in PBS 1X, whereas CDDP and VP-16 stocks were prepared in DMSO. DHMEQ was kindly provided by Dr. Umezawa (Keio University, Japan) (39). Soluble recombinant human TRAIL was purchased from PeproTech Inc. (Rocky Hills, NJ, USA). The NF-κB inhibitor Bay11-7085 [specific inhibitor of IκBα phosphorylation (40) and the mouse anti-β-actin monoclonal antibody were obtained from Calbiochem (San Francisco, CA, USA). Rabbit anti-DR5 polyclonal antibody was purchased from Axxora, LLC (San Diego, CA, USA). The monoclonal mouse anti-YY1 and the HRP-labeled goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology Inc., (CA, USA). The HRP-conjugated goat anti-rabbit IgG and the RPE-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Caltag Laboratories (Burlingame, CA, USA). The monoclonal mouse anti-DR5 and the polyclonal rabbit anti-YY1 antibodies were obtained from Biosource International (Camarrillo, CA, USA) and from Active Motif ( ), respectively. The fluorescein isothiocynate (FITC)-labeled anti-active caspase-3, as well as the FITC and RPE-conjµgated IgG isotype controls were obtained from PharMingen (San Diego, CA, USA).The SureSilencingTM YY1 siRNA kit was purchased from SuperArray Bioscience Corporation (Frederick, MD, USA).

Plasmid Constructs. The DR5 wild type promoter (pDR5 W/T) luciferase reporter plasmid and the pDR5 construct with the 5'-deletion (-605) that includes the YY1 binding site (pDR5/-605) have been previously characterized (29). The pDR5 reporter missing active YY1 binding sequence (pDR5/YY1 mutant) was generated by using the QuikChange site directed mutagenesis method, as previously described by Huerta-Yepez *et al.,* (unpublished data). The NF-κB W/T promoter luciferase reporter plasmid was purchased from InvitroGen……(location?)

6

Cell Treatments. Log-phase PC-3 cells were seeded into 24/well plates at approximately 3.5×10^5 cells/ml and left grown overnight in 0.5 ml complete medium, until the confluence reached the 80%. Prior to each treatment the cell cultures were synchronized for 18 h using medium supplemented with 0.1% FBS. The treatment of PC-3 with the drugs was performed in serum-free conditions for 6, 12, 18 or 24 h. For experiments to measure TRAIL-mediated apoptosis induced by drugs the cells were initially treated with the drugs for 6 h followed by addition of TRAIL and incubation for 18 additional hours.

Luciferase DR5 and NF-κB promoter Reporter Assays. The transfection of PC-3 cells was performed by using the polycationic liposome reagent LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). The transfection was done according to the manufacturer's instructions. Briefly, each of the DR5 promoter constructs: pDR5 W/T, pDR5/YY1 mutant, or pDR5/-605 was first mixed with the liposome reagent in a ratio of 5 µl of LipofectAMINE 2000:1 µg of plasmid in 0.4 ml of serum-free RPMI 1640 for 25 min at room temperature. For the NF-κB promoter construct the relevant ratio was 5 µl of LipofectAMINE 2000:2µg of vector DNA. The resulted 0.4 ml liposome-DNA mixture was then added to each well of cells plated on 6-well plates (70% confluence) with 0.8ml of serum-free medium. 12 h post transfection the transfection medium was removed and fresh medium containing 10% FBS was added to allow the recovery of the cells. After 6 h the cells were treated or left untreated with different concentrations of CDDP, DHMEQ, or Bay11-7085 (3 µM) for 4 or 18 h in serum-free medium. Cells were then harvested in 1X lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. Data were normalized by protein concentration using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Flow Cytometry. To determine the effect of drug treatment on DR5 surface expression as well as on intracellular YY1 protein levels, PC-3 cells were subjected to flow cytometric analysis after each treatment. The cells were detached with PBS-EDTA (1 mM), and washed twice with 1XPBS (Life Technologies, Inc.). For extracellular DR5 staining the cells were resuspended in 100 µl of 1X PBS containing 10% normal human serum (to ensure blocking of the surface Fc Receptors) and incubated for 1h at room temperature. Cells were then washed with 1X perm/wash buffer solution (PharMingen San Diego, CA, USA) and incubated in 100 µl of the same buffer containing 1µl of the mouse anti-DR5 polyclonal antibody at room temperature for 1 h. The samples were subsequently

7

washed once and RPE-conjugated goat anti-mouse IgG was added and incubated with the cells at room temperature for 30 min in light protected conditions. For intracellular YY1 staining the cells, after harvesting, were first permeabalized using 200 µl cytofix/cytoperm solution (PharMingen, San Diego, CA, USA) for 30 min. Following 2 washes with ice-cold 1X perm/wash buffer solution the cells were incubated with 2 µl of rabbit anti-YY1 monoclonal antibody in 100 µl incubation buffer (1X perm/wash buffer solution) at room temperature for at least 3 h. Thereafter, the samples were washed twice with ice-cold 1X perm/wash buffer solution and were stained with RPE-labeled goat anti-rabbit IgG for 30 min at room temperature (light protected). In both assays, after the incubation with the secondary antibodies, the samples were washed once with 1X perm/wash buffer solution and resuspended in 300 µl 1X PBS. Flow-cytometric analysis was performed using Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL) with the System II^{TM} Software and the mean fluorescence intensity (MFI) was recorded. Cells stained with the appropriate RPE-conjugated IgG isotype controls, under the same conditions described above, were served as negative controls.

Determination of Apoptosis. After each treatment, the adherent and the floating cells were recovered by centrifugation at 1500 rpm for 8 min. Afterwards, the cells were washed once with 1X PBS and were resuspended in 200 µl of the cytofix/cytoperm solution for 30 min. Thereafter, the samples were washed twice with ice-cold 1X perm/wash buffer solution and were stained with FITC-labeled anti-active caspase-3 mAb for 1 h (light protected) at room temperature. The samples were subsequently washed once with 1X perm/wash buffer solution and 300 µl of 1X PBS was added prior to flow cytometry analysis on an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). The analysis was performed using the System II software and the percentage of positive cells was recorded. As a negative control, the cells were stained with FITC-conjugated IgG isotype control under the same conditions described above.

PI-based DNA Fragmentation Assay by Flow Cytometry. Apoptosis induced by ADR was determined by DNA staining with PI. Briefly, PC-3 cells were detached with PBS-EDTA (1 mM) and washed twice with 1ml of cold 1X PBS. Supernatant was aspirated, and 1 ml of cold 75% ethanol was added, and cells were incubated at -20^0 C for 1 h. Thereafter, the cells were washed with 1 ml of 1X PBS twice. After the last wash, 150 µl of PI solution [50 µg/ml PI (Sigma, St. Louis, MO) + 0.05 mg/ml Ribonuclease A; Amersham, Life Science International] were added, and the cells were incubated at 37^0 C for at least 30 min prior to analysis and were light protected. DNA analysis was

performed using fluorescence channel 3 (FL-3) in an Epics XL flow cytometer (Coulter Electronics, Inc., Miami, FL). Region markers were drawn for sub-G_0, G_0-G_1, S, and G_2-M populations for quantitation of the cell populations by the flow cytometer. The sub-G_0 population represents the cells containing DNA hypoploidy, a characteristic of apoptotic cells undergoing DNA fragmentation.

Western Blot Analysis. PC-3 cells were incubated for 6, 12, 18 and 24 h in the presence or the absence of drugs in serum-free conditions. The cells were then lysed at 4^0 C in RIPA buffer [50 mM Tris-HCl (PH 7.4), 1% NP40, 0.25% sodium deoxycholate, and 150 mM sodium chloride, supplemented with one tablet of protease inhibitor mixture, Complete Mini (Roche, Indianapolis, IN)]. Protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of Laemmli Sample Buffer (Bio-Rad, Hercules, CA) supplemented with mercaptoethanol. 20 and 30 µg of the cell lysates were then electrophoresed on 12% and 8% SDS-PAGE, respectively and were subjected to Western blot analysis for DR5 and YY1 protein detection. Immunoblots were transferred from the gels onto Hybond nitrocellulose membranes (Amersham, Arlington Heights, IL) using the Trans-Blots SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The nonspecific binding sites were blocked for 1 h at room temperature with freshly prepared 5% nonfat dry milk (Bio-Rad) in TBS/0.1% Tween 20 (Bio-Rad, Hercules, CA, USA) and incubated overnight with the respective antibody at 4^0 C. 1/1000 dilutions of rabbit anti-DR5 polyclonal and mouse anti-YY1 mAb in TBS/0.1% Tween 20 containing 2% nonfat dry milk were used for DR5 and YY1 protein detection, respectively. After washing with TBS/0.1% Tween 20 three times, the membranes were incubated overnight with 1/1000 dilution of HRP-conjugated anti-rabbit IgG at 4^0 C, or incubated with 1/5000 dilution of HRP-conjugated anti-mouse IgG for 30 min at room temperature. Following 3 more washes with TBS/0.1% Tween 20, the membranes were developed with a Lumiglo Western blot detection kit (New England Biolabs, Beverly, MA). Levels of β-actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images by using the AlphaImager software (AlphaInnotec, USA).

Electrophoretic-Mobility Shift Assay (EMSA). Nuclear extract preparations from tumor cells were carried out in 18 h CDDP- treated PC-3 as previously described (38). 15 µg of nuclear proteins were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe YY1 using the EMSA Kit Panomics (Panomics Inc., Redwood City, CA, USA) following the manufacturer's

instructions and as described previously (41). 15µl was subjected to 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-Np (Amersham Pharmacia Biotech, Germany) using the Trans-Blots SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3min. The detection was carried out as per the manufacturer's instructions, after the membranes were exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The relative concentrations were assessed by densitometric analysis.

siRNA Transfections. siRNA transfections were performed by using the X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany) and the SureSilencing YY1 siRNA kit supplied by SuperArray Bioscience Corporation (Fredrick, MD) according to the manufacturers' instructions. Briefly, 3 µl of YY1 siRNA or a negative control of siRNA solution was incubated with 6 µl of the transfection reagent in reduced serum medium OPTI-MEM I (Gibco, Invitrogen Corporation, NY, USA) for 20min to facilitate complex formation. The resulting mixture was added to PC-3 cells (seeded 24 h prior transfection in a concentration of 0.5×10^4 cells/well in a 24-well plate) cultured in 0.45 ml of RPMI 1640 supplemented with 1% FBS. The expression of the surface DR5 receptor as well as the intracellular YY1 protein levels was detected 36 h after transfection using flow cytometry and according to the protocols described above. To determine the effect of YY1 inhibition on PC-3 sensitization to TRAIL-mediated apoptosis, 24 h post transfection, tranfected or untransfected cells were washed once with serum-free RPMI 1640 and treated for 18 h with TRAIL, or CDDP, or the combination of the two in 0.5 ml of serum free medium. Cells were then subjected to anti-active caspase-FITC 3 staining by flow cytometry, as indicated above.

Statistical Analysis. The experimental values were expressed as the mean ± standard error of the mean (SEM) for the number of separate experiments indicated in each case. One way ANOVA test was used to compare variance within and among different groups. When necessary, Student's t-test was used for comparison between two groups. Significant differences were considered for probabilities <5% ($p<0.05$). The statistical analysis was performed using the software SPSS (version: 12).

Synergy. Determination of the synergistic *versus* additive *versus* antagonistic cytotoxic effects of the combination treatment of the PC-3 cells by CDDP, vincristine, ADR, VP-16 and TRAIL was assessed by isobolographic analysis.

RESULTS

Drug- induced sensitization of PC-3 cell line to TRAIL-mediated apoptosis. Sensitization of tumor cells to TRAIL mediated apoptosis has been shown to be induced by a number of chemotherapeutic agents (9, 18, 42, 43). We have investigated the possible mechanism by which chemotherapeutic drugs sensitize TRAIL-resistant prostate tumor cells to TRAIL-induced apoptosis. Human prostate PC-3 cells were used as a model and were treated with subtoxic concentrations of CDDP (1, 3, 5µg/ml), VP-16 (5, 10, 15 µg/ml), ADR (0.25, 0.5, 1 µg/ml), and vincristine (0.01, 0.05, 0.1 µg/ml), used as single agents or in combination with pre-determined concentrations of recombinant TRAIL (5, 10, 20 ng/ml) for 24 h. The cells were then examined for apoptosis by assessing caspase-3 activation or DNA fragmentation by PI staining using flow cytometry as described in the materials and methods The findings demonstrate that, whereas single agents showed moderate cytotoxicity, the combination treatment resulted in significant potentiation of apoptosis and synergy was achieved with all four drugs (Fig. 1) following a TRAIL-dose dependent pattern for all the drug concentrations used (*p* values are presented on the corresponding panels).

Mechanism by which cytotoxic drugs sensitize PC-3 cells to TRAIL-mediated apoptosis.

A. Upregulation of DR5 expression. The mechanism of drug-mediated sensitization to TRAIL was examined. The cytotoxic agents used above, CDDP, VP-16, and ADR, have been reported to upregulate DR5 expression in various cell lines *in vitro* (9, 22, 26, 44, 45). Hence, we examined PC-3 cells treated for 6, 12, 18 and 24 h with each of the drugs at various concentrations, and the cells were subjected to flow cytometry and Western blot analysis for determination of the extracellular and total DR5 protein levels, respectively. Vincristine was additionally included in the list of drugs tested. All treated compared to untreated cells showed a statistically significant increase in DR5 surface expression [(determined as increase in the mean fluorescence intensity (MFI)] for all the concentrations and incubation periods used (Fig. 2) (*p* values indicated on the panels). Time course analyses also revealed a progressive augmentation in DR5 surface expression between the 6th to 18th h of incubation period with CDDP (Fig. 2A), or vincristine (Fig. 2B) (*p*<0.001), while for VP-16 DR5 expression peaked just 12h post-treatment (Fig. 2C). Furthermore, significant concentration-dependent increase in DR5 expression was observed only for vincristine (*p*<0.001).

The total DR5 protein levels were also found substantially elevated after treatment of PC-3 with the above drugs as assessed by Western (Fig. 3). Time kinetic analysis for each drug showed a

time-dependent increase in DR5 expression peaking 24h post treatment with ADR (Fig. 3B), or after 18h incubation with vincristine (Fig. 3C) or CDDP (Fig. 3D), findings similarly to those observed for surface DR5 expression. The above observations were independent of the drug concentration used.

B. Inhibition of both YY1 expression and YY1 DNA-binding activity. The observed upregulation of DR5 expression by the above sensitizing drugs suggested that such agents may inhibit a transcription repressor activity of the DR5 promoter. One binding site for the transcription repressor YY1 has recently been identified in the basic structure of the DR5 promoter (29). Additionally YY1 was recently found to repress Fas transcription (38). We therefore hypothesized that YY1 may negatively regulate DR5 transcription and could be, directly or indirectly, inhibited by the chemo-sensitizing agents. Thus, we examined the protein levels of YY1 in PC-3 cells following treatment with different concentrations of CDDP or ADR for 6, 12, 18 and 24 h. The findings demonstrate a significant decrease in YY1 levels induced by both drugs. The reduction was detected as early as 6h following treatment with the drugs as compared to control (Fig. 4A and 4B respectively). The lowest YY1 protein levels were detected with the highest drug concentration used, although there wasn't a progressive concentration-related reduction.

The effect of CDDP on the YY1 DNA-binding activity was examined by EMSA. A clear CDDP concentration-dependent inhibition of YY1 DNA-binding activity was observed after treatment of PC-3 cells with increasing concentrations of CDDP for 18h (Fig. 4C), indicating the direct, or indirect influence of the drug on YY1 function.

C. Negative regulation of DR5 expression by YY1. The negative transcriptional regulation of DR5 by YY1 was tested by examining a pDR5 W/T luciferase reporter construct and plasmids in which the YY1 binding site was either deleted (pDR5/-605) and/or mutated (pDR5/YY1 mutant). PC-3 cells transfected with pDR5 W/T plasmid expressed a basal luciferase activity and treatment with CDDP significantly augmented the basal luciferase activity in a concentration-dependent manner. PC-3 cells transfected with the pDR5/-605 or pDR5/YY1 mutant constructs resulted in significant potentiation of the basal luciferase activity. In the absence of CDDP, treatment of these transfectants with CDDP didn't reveal any statistically significant enhancement of luciferase activity compared to non treated transfectants (Fig. 5). These findings suggested that YY1 negatively regulates DR5 transcription and that CDDP significantly inhibits YY1 repressor activity. It has been suggested that NF-κB regulates YY1 expression, thus inhibition of NF-κB activity may mimic drug-

induced inhibition of YY1 and subsequent upregulation of DR5 transcription. Indeed, treatment of the cells with the specific NF-κB inhibitor, DHMEQ, resulted in significant augmentation of the luciferase activity comparable to CDDP-mediated activity.

The direct role of YY1 on DR5 expression and regulation of PC-3 sensitivity to TRAIL-induced apoptosis was further examined. PC-3 cells were transfected with a pre-determined concentration of siRNA against YY1 mRNA. To confirm the transfection efficiency, we performed flow cytometry analysis for determination of YY1 protein levels 36 h post-transfection. YY1 protein was found significantly decreased indicating the siRNA-induced inhibition of YY1 expression ($p<0.001$) (Fig. 6A). DR5 surface levels were also assessed by flow cytometry 36h after transfection (Fig. 6B). The comparison between transfected and non-transfected cells or transfected with control siRNA showed significant increase in DR5 expression following inhibition of YY1 by siRNA ($p=0.001$). The above results further confirm the interplay between YY1 suppression and DR5 upregulation.

To examine the role of YY1 in the sensitization of PC-3 to TRAIL-mediated apoptosis, cells transfected with YY1 siRNA were incubated with TRAIL for 18 h and subjected to flow cytometry for apoptosis determination (Fig. 6C). The findings demonstrate a statistically significant induction of active caspase-3 after treatment with a combination of TRAIL and YY1 siRNA compared to treatment with TRAIL alone or YY1 siRNA treatment alone. These findings support the role of YY1 in the regulation of TRAIL-induced apoptosis.

D. Inhibition of NF-κB promoter activity. Drug-induced suppression of the NF-κB/YY1 signaling pathway. The role of YY1 in the underlying mechanism of CDDP-induced cell sensitization to apoptosis via DR5 upregulation was further confirmed by upstream activators of the YY1 signaling pathway. NF-κB has been shown to be involved in the regulation of tumor cell survival and growth and in the transcriptional activation of several anti-apoptotic genes (46-48). Using a wild type NF-κB promoter luciferase reporter assay, we show that CDDP reduces the NF-κB promoter activity in transfected PC-3 cells in a concentration-dependent manner (Fig. 6D). The direct role of NF-κB inhibition in CDDP-mediated DR5 upregulation was corroborated by the use of the NF-κB chemical inhibitor Bay 11-7085 which specifically inhibited the promoter activity in our reporter system (Fig. 7). Previous findings have shown that Bay 11-7085 was also capable of sensitizing tumor cells to both drug and TRAIL-mediated apoptosis, like CDDP (data not shown). Significant inhibition of NF-κB

14

activity was already established after only 4h incubation of the cells with increasing concentrations of CDDP, indicating the early drug-induced suppression of YY1 activity.

In summary, our present findings demonstrate the negative regulation of DR5 expression by the transcription repressor YY1, and suggest that inhibitors of YY1 like CDDP, upregulate DR5 expression and sensitize tumor cells to TRAIL-mediated apoptosis.

DISCUSSION

Recent studies have indicated that the sequential combination treatment of subtoxic concentrations of chemotherapeutic drugs and TRAIL could overcome TRAIL resistance and triggers apoptosis in resistant cells (49). However, the mechanism of reversal of TRAIL resistance remains unclear. Several factors might modulate the sensitivity of cells to TRAIL, such as degradation of c-FLIP (50)), Bax reactivation and Smac/DIABLO release in MMR or Bax-deficient tumors (51), as well as p53 downstream transcriptional target genes such as DR5. DR5 has been shown to contribute significantly to TRAIL sensitization (52, 53). Since DR5 has been considered to be a major death receptor on most tumor cells (54) overexpression of DR5 may contribute to tumor cell sensitization to apoptosis mediated by TRAIL.

In the present study we showed that the DNA-damaging chemotherapeutic drugs, CDDP, ADR, etoposide, as well as the inhibitor of microtubule formation, vincristine, elicited increased expression of DR5 in the human androgen-independent and p53-deficient prostate cell line PC-3. Untreated cells were relatively resistant to TRAIL and drug-induced DR5 expression by itself did not lead to significant apoptosis. However, treatment with soluble human TRAIL in combination with the above drugs caused a synergistic cell death through TRAIL-receptor interaction and caspase activation. Apoptosis induction in response to most DNA-damaging drugs usually requires the function of the tumor suppressor p53. However, conventional treatment eventually selects for tumor cells in which p53 is inactivated, resulting in resistance to therapy. One of the attractive features of TRAIL is its ability to induce apoptosis in a variety of tumor cell lines regardless of p53 status, particularly in cells in which the p53 response pathway has been inactivated, thus helping to circumvent resistance to chemotherapy (43, 55). In tumors that have lost p53, DR5 triggering by agents that upregulate DR5 independently of p53, might lead to synergistic apoptosis activation in TRAIL-based therapies , as well as reduce the probability that tumor cells will become resistant to TRAIL (55).

Genotoxic agents like anthracyclines (ADR) or etoposide are generally known to induce the expression of DR5 in a p53-dependent or -independent manner (21, 22, 56). Glucocorticoids such as dexamethasone or IFN-γ have been shown to induce p53-independent upregulation of DR5 in colon cancer cells (23). DNA topoisomerase I inhibitors like β-lapachone upregulate DR5 in a p53-dependent manner in the above tumor type (23), however it could induce apoptosis to leukemia and prostate cancer (57), glioma (58)), breast cancer (59) and hepatoma cells (60) independently of p53.

Betulinic acid, a cytotoxic chemotherapeutic compound induces DR5 expression in colon cancer, melanoma and glioblastoma cell lines independent of p53 status (23). In addition, IFN-α (61), bile acids (62), as well as CDDP (22, 42) have also been recently reported to be potent inducers of DR5 in different human malignant cells.

In order to investigate possible underlying mechanisms of cell sensitization to TRAIL after treatment with genotoxic drugs, we hypothesized that the observed DR5 upregulation by the drugs might derive from partial inhibition of pathways that negatively regulated DR5 suppression. This hypothesis was tested and we showed, by a number of indirect and direct lines of evidence, that inhibition of the transcription factor YY1 significantly contributed to DR5 overexpression. YY1 has been shown to regulate the transcriptional activity of a series of gene promoters, either acting as activator, or repressor (63). This transcriptional dual function may be mediated either by YY1 interaction with other cellular transcription factors including TBA, TAFs, TFIIB and Sp1, or by recruitment of histone modification enzymes including p300, HDACs and PRMT1 (64). In our system YY1 protein was found substantially decreased after treatment of PC-3 with CDDP or ADR and was accompanied by increased surface and total DR5 protein levels. YY1 DNA-binding activity was also found decreased after treatment with CDDP, while introduction of siRNA against YY1 mRNA in PC-3 resulted again in DR5 overexpression and sensitized the cells to TRAIL-mediated apoptosis. The direct involvement of YY1 in DR5 transcriptional regulation and subsequently its contribution to apoptotic signaling via DR5 was established by using a DR5 promoter luciferase reporter system whereby the YY1 binding domain (29) was either mutated, or deleted. The DR5 promoter activity was found significantly elevated in cells transfected with the above plasmids compared to basal luciferase activity observed in pDR5 wild type transfectants. Treatment with CDDP also induced enhanced promoter activity only in wild type transfectants. The above findings support a suppressive role of YY1 in DR5 transcription and introduce a new target for alternative therapies which are intended in the reversal of tumor cell resistance to TRAIL.

Previous findings in prostate models demonstrated that Fas expression was under the negative regulation of NF-κB via the transcription repressor YY1 (38). In terms of NF-κB involvement in DR5 regulation, recent studies have proposed the differential roles of RelA (p65) and c-Rel subunits of NF-κB in DR4 and DR5 expression and in apoptosis. In particular, overexpression of RelA or a transcriptional deficient mutant of c-Rel by TRAIL have been shown to inhibit DR4 and DR5

expression, while overexpression of c-Rel enhances DR4, DR5, and Bcl-Xs and inhibits c-IAP1, c-IAP2 and survivin again after TRAIL treatment (28, 65). The mechanism of NF-κB-mediated DR5 regulation is, however, unknown. It has been demonstrated that the etoposide-induced DR5 expression requires the first intronic region of the DR5 gene and mutation of a putative NF-κB binding site in this intron eliminates DR5 promoter activity (66). Furthermore, an involvement of HDAC1 in the differential regulation of DR5 by NF-κB has also been proposed (67). Since the NF-κB direct or indirect implication in the DR5 regulation has been well established by the above studies and additionally putative DNA binding sites for both YY1 and NF-κB factors have been identified by Yoshida *et al.* in the DR5 promoter region, it was of interest to further determine whether a NF-κB/YY1 pathway is also implicated in DR5 upregulation and apoptosis induction after drug treatment in our system.

Palayoor *et al.,* have reported that NF-κB is constitutively activated in the hormone-refractory prostate cancer cell lines PC-3 and DU145 (68). The suppression of NF-κB survival signaling by various agents has been shown to sensitize different neoplasms to the anti-tumor effects of TRAIL. Nitrosylcobalamin (NO-Cbl), an analog of vitamin B12 (46), or other nitric oxide donors (69)) and IFN-α (61) have been reported to inactivate NF-κB via different mechanisms including decreased phosphorylation of IκBα, inhibition of NF-κB DNA binding activity, or RelA inactivation in RelA/p50 heterodimers. Other nonsteroidal anti-inflammatory drugs, like Dexamethasone or Ibuprofen could also inhibit the activation of NF-κB by activation of intracellular IκB synthesis, or inhibition of an upstream regulator of IκB kinase respectively (70, 71).

The reports on the effect of genotoxic drugs, or other groups of anticancer agents on NF-κB activity remain conflicting. Many antineoplastic agents including anthracyclines (daunomycin and doxorubicin), paclitaxel and vinca alkaloids (vincristine and vinblastine) have been shown to induce NF-κB activation in human lung adenocarcinoma cell lines via activation of protein kinase C resulting in IκBα degradation (7). Regulation of vinca alkaloid or doxorubicin-induced apoptosis by activation of NF-κB/IκB pathway has also been reported in several human tumor cells (72, 73). NF-κB activation has been considered to be a side effect of many commonly used chemotherapeutic drugs, which may blunt the therapeutic efficacy of these compounds. However, a recent study (74) focused on the effect of doxorubicin on NF-κB activation on breast cancer cells has shown that doxorubicin treatment activates NF-κB signaling and produces NF-κB complexes that are competent for NF-κB binding *in*

vitro. These NF-κB complexes suppress, rather than activate, constitutive- and cytokine-induced NF-κB-dependent transcription. The same study also showed that doxorubicin treatment produced RelA, which was deficient in phosphorylation and acetylation which in turn blocks NF-κB signaling in a histone deacetylase-independent manner. Furthermore, the activated NF-κB was characterized by reduced binding stability to κB elements *in vivo*. In the same line of chemotherapeutic drug-induced NF-κB inactivation are reports indicating that CDDP was able to cause a decrease in the phosphorylation of IκBα and activity of NF-κB in cisplatin-sensitive ovarian cancer models (6).

In our system, transfection of PC-3 with a wild type NF-κB luciferase reporter plasmid and subsequent treatment with CDDP resulted in early reduction of luciferase activity which was as significant as the one observed after cell treatment with the NF-κB inhibitor Bay11-7085. Although there is no supportive evidence from other studies for direct drug influence on NF-κB transcriptional activation, we can not exclude the possibility that in our system the drugs can act with similar ways as those described above. Thus CDDP or ADR, the main chemotherapeutic agent used for prostate tumors, may mediate their action at the NF-κB signaling pathway, resulting in reduction of NF-κB-dependent genes' expression in cancer cells. However, it will be of great interest to examine in our model the expression status of the NF-κB subunits related to DR5 regulation, after treatment.

In this study, inhibition of YY1 DNA-binding activity or YY1 protein expression by either drug treatment or after transfection with YY1 small interfering RNA resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. Despite absence of evidence in the literature for direct transcriptional regulation of YY1 by NF-κB it has recently been reported in B cell lymphoma cell lines an association of YY1 with RelB, but not with other NF-κB family members (75). In some gene promoters like serum amyloid A (SAA1) gene promoter has also been demonstrated an antagonistic and inhibitory role of YY1 to NF-κB binding (76). Recently we demonstrated that the regulation of Fas resistance by NF-κB is mediated via YY1 expression and activity in a Non-Hodgkin's Lymphoma cell line, whereas chemoresistance by NF-κB is mediated by Bcl-x(L) expression (77). Consistent with the above reported data, our findings suggest three possible mechanisms underlying the drug induced sensitization of PC-3 to TRAIL-mediated apoptosis. These mechanisms may be referred either in direct inhibition of NF-κB activity and function by genotoxic drugs, via the ways described above, or direct inhibitory effects of drugs on YY1 expression and DNA binding activity, such as induction of YY1 S-nitrosylation which has been observed recently in PC-3 after

DETANONOate treatment (78). Both mechanisms may lead to DR5 upregulation and apoptosis induction by TRAIL. Alternatively, the drug-induced inhibition of NF-κB may result in inhibition of YY1, as an NF-κB-dependent gene, which in turn confers to DR5 overexpression and cell sensitization to TRAIL-induced apoptosis.

In any case the involvement of NF-κB and YY1 as independent or cooperative regulators of DR5 expression seems to play an important role in the mechanism of drug-induced cell response to TRAIL-mediated apoptotic signaling in prostate models. YY1 overexpression is considered to be an important malignant marker, as it has been found in several tumors including prostate (79) and multiple myeloma (unpublished data) tissues. Overexpression of YY1 may also regulate resistance and inhibit tumor cell destruction by the host immune system which may lead to tumor progression. By identifying the role of YY1 in the mechanism of drug-mediated tumor sensitization to TRAIL-induced apoptosis, YY1 could be considered as a new target for antitumor therapeutic approaches which in combination with other therapies may result in reversal of tumor chemo- or immunoresistance.

Abbreviations

The abbreviations used are: ADR, Adriamycin; CDDP, *cis*-diammine-dichloro-platinum; CTL, Cytotoxic T lymphocytes; Dc R,decoy receptor; DETANONOate, (Z)-1-[2-(2 aminoethyl)-N-(2-ammonio-ethyl0amino] diazen-1-ium-1,2-diolate; DHMEQ, dehydroxymethylepoxyquinomicin; DMSO,   ; DR, death receptor; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; HDACs, histone deacetylase; HRP, horseradish peroxidase; IFN, Interferon; MMR, mismatch repair; NF-κB, nuclear factor-κB; PBS, phosphate- buffered saline; PC, prostate cancer; PI, propidium iodide; RIPA, radioimmunoprecipitation assay (buffer); RPE, R-Phycoerythrin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; TNF; tumor necrosis factor; TBS, Tris- buffered saline; TRAIL/Apo-2, TRAIL-related apoptosis-inducing-ligand; VP-16; etoposide.

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Figure legends

Figure 1. Sensitization of PC-3 cells to TRAIL-mediated apoptosis after treatment with the indicated concentrations of vincristine (Fig. 1A), VP-16 (Fig. 1B), CDDP (Fig. 1C) and ADR (Fig. 1D). PC-3 cells were seeded in 24 well plates and subjected to single drug treatment for 6hrs, followed by 18 h treatment with 5, 10 and 20 ng/ml TRAIL where appropriate. Flow cytometric analysis for active caspase-3 (Fig. 1A, B, C), or for propidium iodide-based DNA fragmentation (Fig. 1D) was performed in all samples. Apoptosis was determined either as % of cells expressing active caspase-3, or as % of Sub-G_0 population assessed after PI staining. Our findings demonstrate that only the combination treatment resulted in significant potentiation of apoptosis and synergy was achieved for all the drugs used, as indicated by the isobolographic analysis (Fig. 1E, F, G, H). The data represent the mean±SEM of at least three independent experiments. *p value: single drug or TRAIL treatment *vs* combined treatment, **p value: TRAIL dose dependent increase in caspace-3 activation or sub-Go population, for each drug concentration used, ***p value: single ADR or TRAIL (10 or 20 ng/ml) treatment *vs* combined treatment.

Figure 2 Time kinetic analysis of surface DR5 expression in PC-3 cells treated with cytotoxic drugs. PC-3 cells were incubated with the indicated concentrations of CDDP (Fig. 2A), vincristine (Fig. 2B), or VP-16 (Fig. 2C) for 6, 12, 18 and 24 h. DR5 surface expression was assessed using flow cytometry analysis for each time point. The data represent the observed mean fluorescence intensity (MFI). Our results revealed a time-analog augmentation in DR5 expression picking 18 h post-treatment with CDDP (Fig. 2A), or vincristine (Fig. 2B). An earlier pick, regarding the DR5 overxpression, was observed in cells treated with VP-16 (12 hr) (Fig. 2C). Differences in dose-dependent responses were also observed among the drugs used. Data represents the mean±SEM of one experiment in triplicates. *p value: untreated *vs* drug treated cells for each time point, **p value: time (6-18 hr) dependent increase in DR5 expression for each drug concentration used.

Figure 3 Time course analysis of drug-induced DR5 protein upregulation, as assessed by Western blot. PC-3 cells were treated or left untreated with VP-16 (Fig. 3A), ADR (Fig. 3B), vincristine (Fig. 3C), or CDDP (Fig. 3D), in increased concentrations for 6, 12, 18 and 24 h. Total cellular protein was extracted and separated by SDS-PAGE and transferred onto nitrocellulose membrane as

described in materials and methods. The membrane was stained with anti-DR5 mAb. Levels of β-actin were used to normalize the protein expression by densitometric analysis. The blots represent one of three independent experiments. Despite differences in time or drug dependent responses, all drug-treated cultures showed a significant DR5 overexpression compared to untreated cells for each time point.

Figure 4   Treatment of PC-3 cells with ADR (Fig. 4A), or CDDP (Fig. 4B) results in the YY1 protein downregulation and inhibition of YY1 DNA binding activity (Fig. 4C). For YY1 protein determination the cells were incubated with the indicated drug concentrations for 6, 12, 18 and 24 h and the extracted protein lysates were subjected to Western blot analysis. Relative YY1 protein levels were determined by densitometry and expressed in arbitrary units compared to β-actin levels (internal control). The blots are representative of one out of three separated experiments. For both drugs the greater YY1 protein decrease was observed in the higher drug concentration used. For the determination of YY1 DNA binding activity nuclear extracts were prepared from PC-3 cells treated with different concentrations of CDDP for 24 h. Extracts from untreated cells were also served as control. 15 μg of the whole extract mix was subjected to EMSA as described above, and YY1 DNA binding activity was assessed. Relative YY1 DNA binding activity was determined by densitometry and expressed in arbitrary units compared to control. Our findings demonstrate a significant inhibition in YY1 DNA binding activity induced by all CDDP concentrations used. Data from one representative experiment.

Figure 5   Negative regulation of DR5 transcription by YY1 and CDDP-induced upregulation of DR5 promoter activity. PC-3 cells were transfected with 1 μg of pDR5 luciferase reporter plasmids (pDR5 W/T, pDR5/YY1 mutant or pDR5/-605) for 24 h. After transfection the cells were treated or left untreated with 1 or 3 μg/ml of CDPP or DHMEQ for 18 h. The promoter activity was determined by assessment of luciferase activation expressed as relative light units. Our data show that PC-3 cells transfected with DR5 W/T plasmid resulted in basal luciferase activity (control) and treatment with CDDP significantly augmented luciferase activity in a dose-depended manner. PC-3 cells transfected with the deleted, or YY1 mutated constructs also resulted in significant potentiation of the basal luciferase activity. However treatment of those transfectants with CDDP didn't reveal statistically

significant enhancement of luciferase activity compared to non treated transfectants. DHMEQ also augmented the basal luciferase activity in the pDR5 W/T – transfected cells in a dose dependent manner, however as observed with CDDP, it didn't change significantly the DR5 promoter activity in any of the other transfectants. The values represent the % of control and are the mean±SEM of 7 independent experiments. *$p<0.03$, ** $p\leq0.016$, *** $p<0.001$, control *vs* pDR5/-605 or pDR5/YY1 mutant - transfected and treated cells.

Figure 6   Induction of DR5 overexpression and sensitization to TRAIL-mediated apoptosis by YY1 siRNA. PC-3 cells were transfected or left untransfected with 3 µl siRNA against YY1 mRNA, or control siRNA (negative control). After 36 hrs the efficiency of the transfection was determined by measuring the YY1 protein levels by flow cytometry (Fig. 6A). A significant reduction in YY1 protein levels was obsverved indicating the specific inhibition of the YY1 expression by siRNA. 36 h post-transfection the surface expression of DR5 was also assessed by flow cytometry (Fig. 6B). The findings reveal DR5 upregulation induced by suppression of YY1 expression. The flow cytometry data regarding YY1 and DR5 protein evaluation represent the mean fluorescence intensity (MIF) and are the mean±SEM of two independent experiments. *p value: untreated *vs* YY1 siRNA treated cultures. For determination of cell sensitization to TRAIL-mediated apoptosis after transfection, 24 h transfected PC-3 cells were treated or left untreated with 1, 2.5 or 5 ng/ml recombinant TRAIL for 18 h. Thereafter they were stained with FITC-labeled anti-active caspase-3 and apoptosis assessed by flow cytometry (Fig. 6C).Our results indicate a statistically significant increase in caspase-3 activation after combination treatment of cells with TRAIL and YY1 siRNA. **p value: single TRAIL or YY1 siRNA treatment *vs* combined treatment. CDDP was also able to induce inhibition of NF-κB promoter activity (Fig. 6D). PC-3 cells were subjected to 24 h transfection with an NF-κB W/T promoter reporter construct followed by treatment with CDDP (1 or 3 µg/ml) or Bay11-7085 (3 µg/ml) for 4 or 18 h. NF-κB promoter activity was determined by induction of luciferase activity expressed as relative light units. Untreated cells were served as control. The findings reveal a statistical significant CCDP-induced inhibition of NF-κB promoter activity mainly observed after 4 h treatment with CDDP. Bay11-7085, an inhibitor of NF-κB, was used as positive control of NF-κB inhibition. The data represent the mean±SEM of four independent experiments. *$p\leq0.05$, **$p=0.004$, ***$p\leq0.001$ transfected *vs* cells transfected and treated.
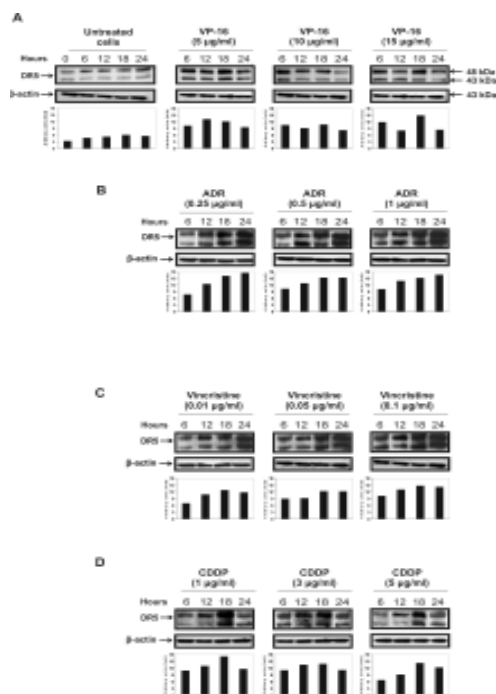
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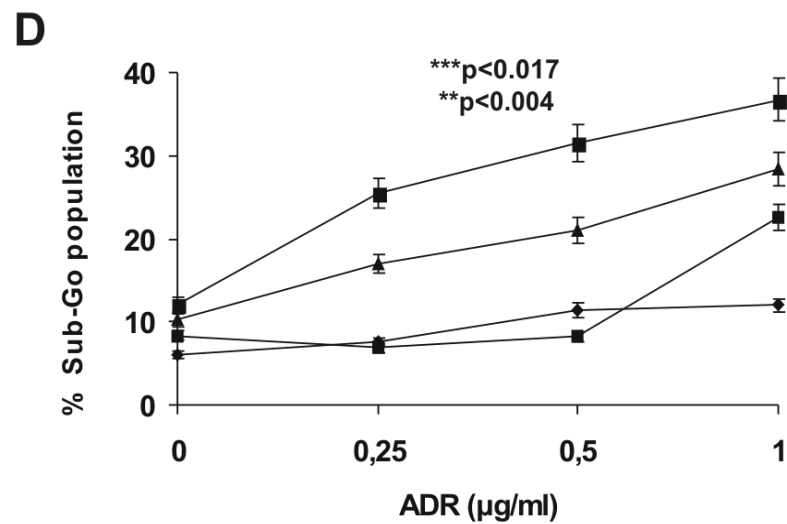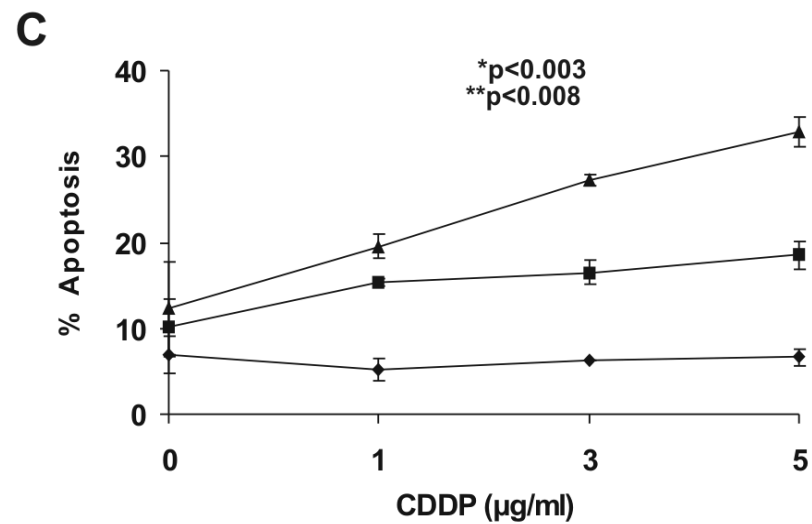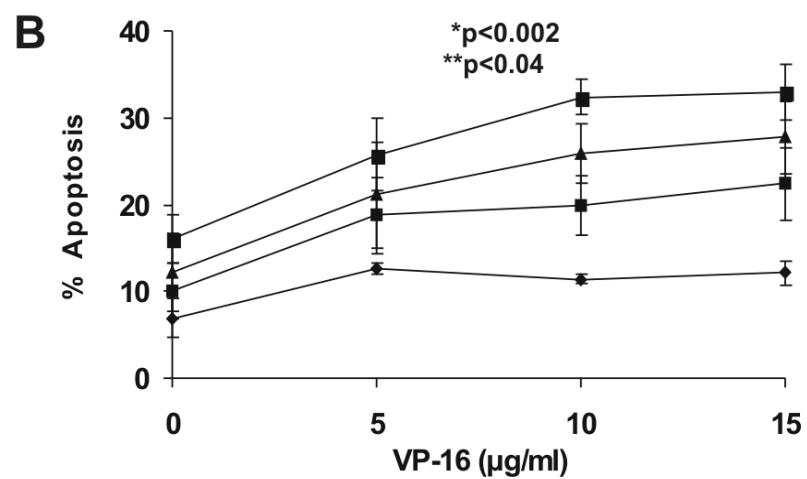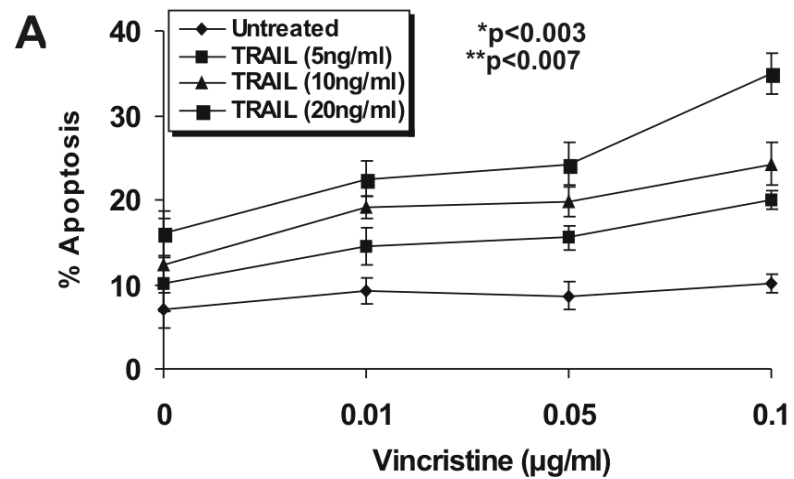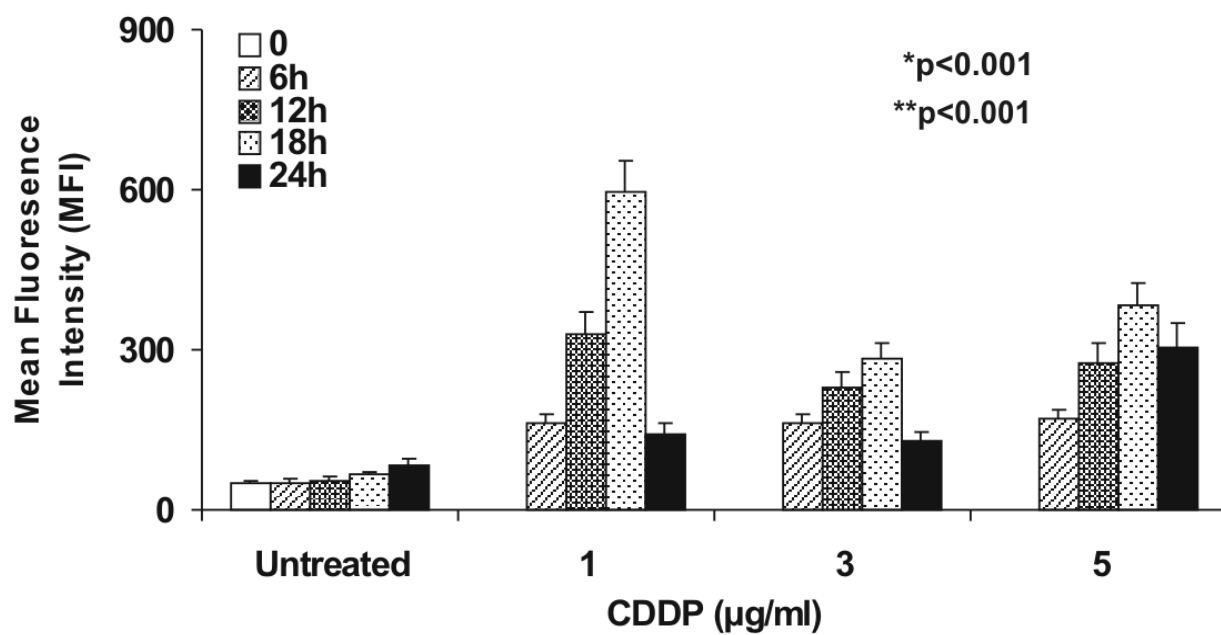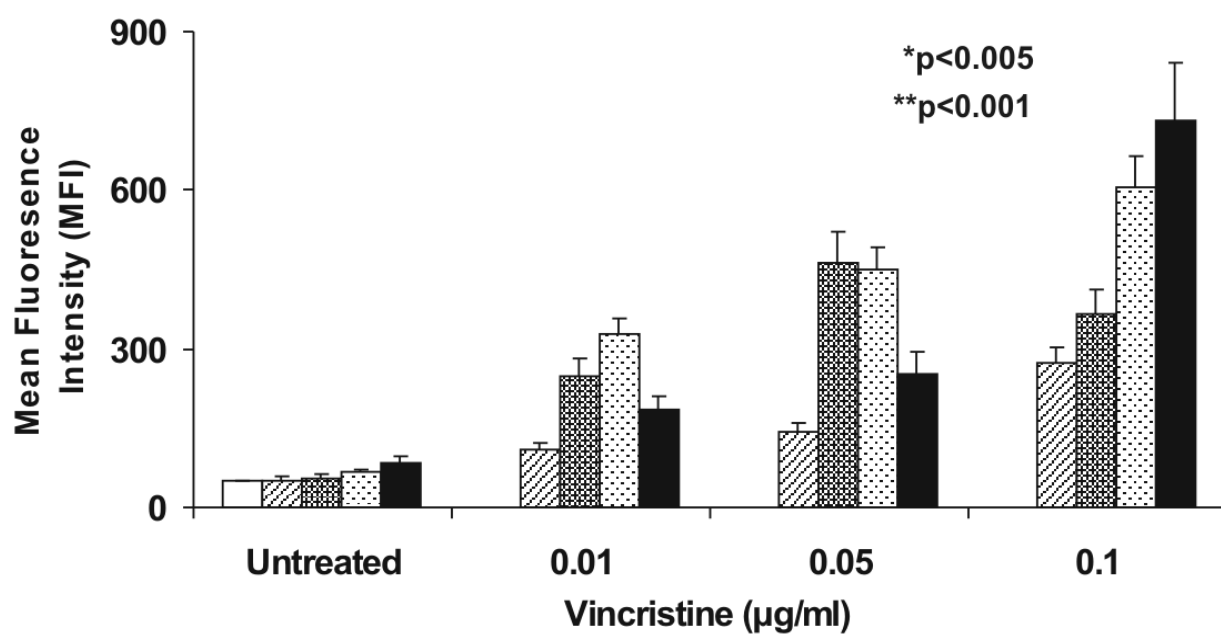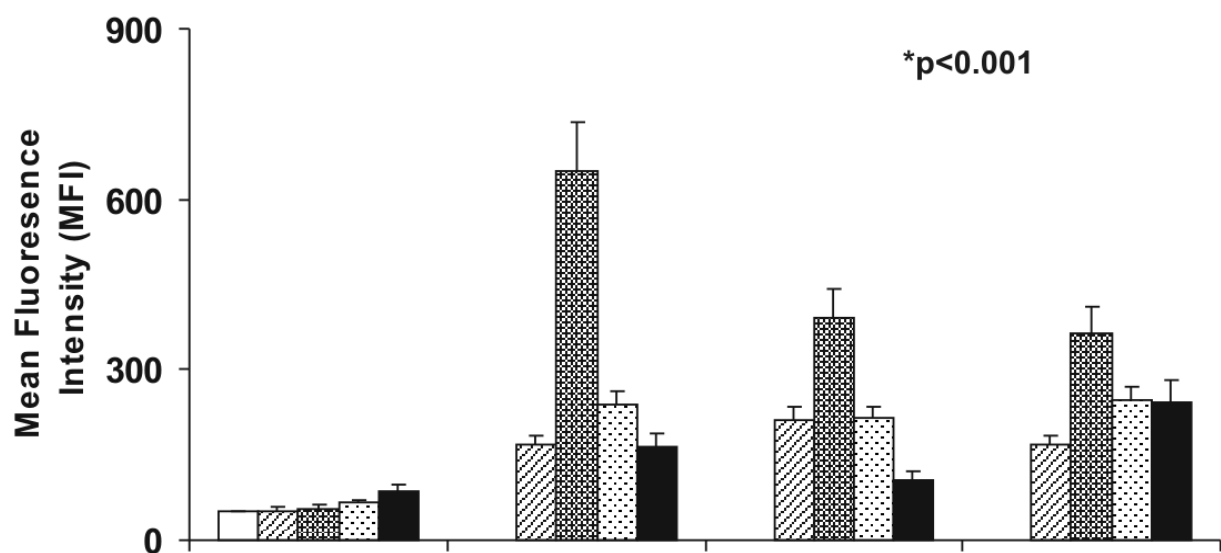
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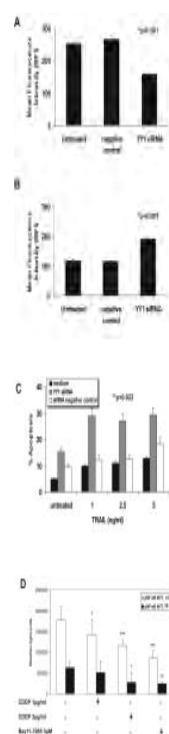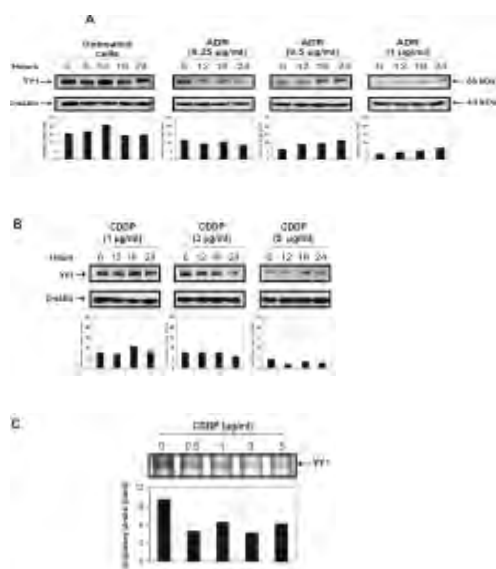

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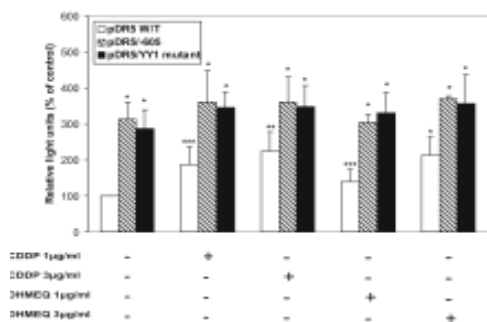Figure 5

October 2, 2005
Ben's Version

Overexpression of X-Linked Inhibitor of Apoptosis Protein (XIAP) in Human Prostate Cancer

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Running Title: XIAP in prostate cancer

Word Count: _____

Keywords: XIAP, X-Linked Inhibitor of Apoptosis Protein, tissue microarray, prostate cancer, diagnostic maker, tumor marker, prognostic indicator.

Abbreviations: Bcl2 B-Cell CLL / Lymphoma-2; XIAP, X-Linked Inhibitor of Apoptosis Protein; PIN prostatic intraepithelial neoplasia; BPH benign prostatic hyperplasia; H & E, Hematoxylin and Eosin; ABC avidin-biotin complex; TMA, tissue microarray; PSA, prostate-specific antigen. CASPASE cysteine-containing aspartate-specific proteases; Fas-associated death domain (FADD); SMAC/DIABLO second mitochondria-derived activator of caspase/ direct IAP-binding protein with low pI; MMP mitochondrial membrane permeability; TRAIL TNF-related apoptosis-inducing ligand; APAF1 apoptotic protease activating factor 1; BID BH3 Interacting domain Death agonist; IAP

inhibitors of apoptosis; NAIP neuronal apoptosis inhibitory protein; ML-IAP melanoma inhibitor of apoptosis

ABSTRACT
Objective: The X-linked Inhibitor of Apoptosis (XIAP), a member of the family proteins, has been linked to tumor cell survival and drug resistance by direct blockade of caspase-mediated ~~extrinsic~~ apoptotic pathways. Thus, XIAP status may help predict prostate cancer recurrence and clinical response to therapies relying on unencumbered apoptotic machinery. It is therefore important to validate the foundational protein expression patterns of XIAP and examine its prognostic implications in human prostate cancer.

Methods: Immunohistochemistry was performed on tissue microarrays constructed from paraffin embedded primary prostate cancer specimens from 226 hormone naïve patients who underwent radical retropubic prostatectomy. 223 cases provided informative epithelium for XIAP analysis encompassing 1,107 total tissue microarray spots including morphologically normal prostate (NL; n=252), benign prostatic hyperplasia (BPH; n=122), prostatic intraepithelial neoplasia (PIN; n=48) and invasive prostate cancer (Cancer; n=685). XIAP expression was scored in a semi-quantitative fashion using an integrated intensity measure (0.0-3.0). The protein expression distribution was examined across the spectrum of epithelial tissues and its association with standard clinicopathological covariates and tumor recurrence was examined in 192 outcome-informative patients.

Results: The mean XIAP expression was significantly higher in prostate cancer (intensity = 1.32) compared to PIN (intensity = 1.08; p=0.019), normal (intensity = 0.78; p<0.0001), and BPH (intensity = 0.57; p<0.0001). 69% of BPH stained negatively to weakly (intensity <1.0), 53% of normal, 37% of PIN and only 26% of prostate carcinomas. With XIAP expression dichotomized at an intensity of 1.8, XIAP is an independent predictor of tumor recurrence in multivariate Cox proportional hazards analysis in all patients (P = 0.0025; HR = 8.92; 95% CI = 2.16-38.86), as well as after substratifying by Gleason score (P = 0.010; HR = 6.61; 95% CI = 1.57-27.89 for high Gleason score [7-10] cases). In patient substrata with low Gleason score tumors [2-6], no patients (0%) with an XIAP intensity > 1.8 (n=23) experienced tumor recurrence, while 26% with low XIAP (n=89) recurred. Patients with high grade or non-organ confined tumors with high XIAP have a lower risk of recurrence as a group than any patients whose tumors express low XIAP, even those of low grade or that are organ confined. These data are consistent with findings in a recent report (Krajewska 2003).

Conclusions: XIAP is expressed at higher levels in prostate cancers compared to matched normal tissues. High XIAP expression is strongly associated with a reduced risk of tumor recurrence, and is not directly associated with Gleason score, tumor stage, capsular involvement or preoperative PSA status, suggesting that it is a novel prognosticator and a potential target for prostate cancer diagnosis and therapy. Based on these results, patients with lower XIAP expression in tumors are most in need of therapeutic intervention and may also be most responsiveness to chemotherapeutic and death receptor targeted therapies.

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and ranks second among all malignant cancers in men with an estimated 232,090 new diagnoses and 30,350 deaths in the US in 2005 (Jemal 2005). Most prostate cancers are clinically localized or regional upon diagnosis and patients enjoy a 5-year survival rate approaching 100% (American Cancer Society, 2005). Nonetheless, as evidence of the slow but steady nature of this disease, 30-40% will experience PSA recurrence within 10 years following definitive surgery or radiation treatment (Han 2003). Patients with high risk or advanced disease on staging workup, or who have recurred, historically receive treatment with exogenous or endogenous androgen ablation, sometimes supplemented with chemotherapy and/or radiation (Bolla 2002; Hanks 2003; Pilepich 2003). Unfortunately, progression of tumor cells to therapy resistance inevitably ensues, leaving few alternatives to care. As a result, the median survival in advanced disease is only 18-20 months with an overall survival of 24-36 months (XXXX).

Apoptosis (programmed cell death) is an important mechanism in tissue development, homeostasis, and response to stress factors. It relies on a concerted and tightly balanced signaling pathway involving pro- and anti-apoptotic proteins. Dysregulation of apoptosis is a major contributor to tumorigenesis (Bilim 2003; Krajewska 2003), tumor growth (Denmeade, 1996), progression (Krajewska 2003), metastases (Berezovskaya, 2005) and resistance to conventional therapies (Holcik 2000; Tamm 2000; Asselin 2001, Holcik 2001A; Imoto 2001). The key executor of the apoptotic pathway is a group of cysteine aspartyl-specific proteases, known as caspases, that are present in the cytosol as inactive zymogens and are proteolytically activated by the appropriate apoptogenic agents (Cryns Review 1998; Thornberry Review; 1998; Shi, 2002). Once activated, caspases cleave key structural proteins as well as proteins involved in DNA repair and cell signaling, with resultant cell death (Shi, 2002). The caspase cascade is most commonly initiated either through the extrinsic pathway involving a number of receptor families, commonly called death receptors, or through the intrinsic pathway where various stress-inducing stimuli facilitate apoptosis utilizing a mitochondrial-dependent mechanism.

Death ligands, including TNF-alpha, FAS L and TRAIL, bind to their respective death receptors and form the death inducing signaling complex (DISC) with procaspase 8 and FADD (Ashkenazi 1998; Reed 2000B). Caspase-8 subsequently autocatalytically initiates its own activation followed by cleavage of downstream caspases into activated states. The mitochondrial pathway is activated by physiological stress, including that induced by conventional cancer therapies, and is activated by p53 after DNA damage ultimately resulting in increased mitochondrial membrane permeability (MMP) and release of a variety of apoptogenic proteins, most notably cytochrome c, Smac/DIABLO and HtrA2/Omi (XXXXX). Cytosolic cytochrome c forms an apoptosome complex (Cain 2002) with pro-caspase 9 and Apaf-1, which in turn releases active caspase 9. Like the extrinsic pathway, the intrinsic pathway converges on activation of caspase 3 (van Gurp 2003). Caspase 8 stimulation by the DISC formation may also facilitate degradation of mitochondrial proteins through caspase 8-induced cleavage of the Bcl-2 family member

BID. Upon cleavage, a fragment of BID is translocated into the mitochondria constituting an alternate pathway to MMP.

Tight regulation of caspase activation is required to prevent unchecked cell death. To this end, members of the inhibitors of apoptosis (IAP) protein family provide an intrinsic layer of anti-apoptotic regulation. IAP's are an evolutionarily conserved protein family that functions to block cell death by binding to and inhibiting caspases (Uren, 1996; Deveraux, 1997; Deveraux, 1999; Holcik, 2001A; Holcik, 2001B). The IAP family member proteins were originally described in baculovirus (Crook 1993; Birnbaum 1994; Duckett 1996) and are characterized by having 1 to 3 highly conserved 70 amino acid zinc finger motif regions called baculovirus inhibitory repeat (BIR) domains that directly bind and inhibit active caspases (Crook 1993; LaCasse 1998). Eight human IAP's have been reported, namely XIAP, cIAP1, cIAP2, Survivin, NAIP, Apollon, Livin, and ILP-2 (Reed, Review 2001).

The X-linked inhibitor of apoptosis, XIAP, is the best characterized of the IAP family members in terms of its potent caspase inhibitory mechanisms, and is considered the prototype of the IAP protein family (Deveraux 1997; Holcik 2001). The XIAP protein is a 497-amino acid polypeptide encoded on chromosome Xq25 (Rajcan-Separovic 1996). It contains 3 BIR domains; BIR2 and linker domains binding and potently inhibiting caspases 3 (Reidl 2001; Suzuki, 2001) and 7 (Chai 2001; Huang 2001; Suzuki, 2001) through steric hindrance, while binding of caspase 9 to BIR3 prevents its catalytic activity through maintenance of its monomeric state (Deveraux 1997; Deveraux 1999; Ekert 2001; Huang 2000; Riedl 2001; Shiozaki 2003; Srinivasula 2001). Thus, XIAP can antagonize both the intrinsic and extrinsic apoptotic pathways (Hengartner, 2000). No function is yet attributed to the first BIR domain (Scott 2005). Studies have shown that overexpression of one or more members of the IAP family may result in loss of the expected apoptotic growth-control response to pro-apoptotic triggers, including activation of death receptors, chemotherapy or radiation (Amanatullah, 2000; Holcik and Korneluk 2001B; Holcik 2001A; Visakorpi 2003). Indeed, abundant XIAP protein expression has been reported in a number of human cancers, including leukemia (Tamm 2000; Byrd 2002; Carter 2005, Nakagawa 2005), lymphoma (Kashkar 2003), and tumors derived from prostate (Krajewska 2003; Liu 2004; Berezovskaya 2005; Schimmer 2004; Schimmer, 2004), colon (Krajewska 2005), lung (Ferreira 2001A; Hoffman 2002), cervical (Liu 2001), bladder (Bilim 2003), hepatocellular (Shiriki 2003), and vascular (Levkau 2001) cells. Abundant XIAP is also seen in many of the NCI-60 cancer cell lines (Fong 2000; Tamm 2000).

As evidence of XIAP's influence on therapy resistance, down-regulation of XIAP has been shown to induce apoptosis and reduce chemoresistance in ovarian, (Sasaki 2000), gastric (Tong 2005), lung (Hu, 2003) and prostate cancer cells (Amantana, 2004; Schimmer 2004A).

Below Paragraph is for Discussion:
As a result of both apoptotic inhibition and treatment resistance, high XIAP expression is often associated with poor patient outcome linked to increased tumor cell survival (Tamm 2000; Tamm 2004A; Tamm 2004B; Ramp, 2004; Yan, 2004; Krajewska 2005; Muris 2005) However, a number of studies find that XIAP expression lacks significant prognostic association (Ferreira 2001A; Liu 2001; Carter 2003A; Krajewska 2005), while in prostate (Krajewska, 2003) and lung cancers (Ferreira 2001B), high XIAP protein expression has been associated with improved outcome. Therefore the clinical implications of XIAP are controversial and require further validation studies.

Human prostate cancers typically exhibit slow growth kinetics, resulting in potentially suboptimal effectiveness of cytotoxic agents (Denmeade, 1996). Because of this, and the frequent dysregulation of apoptosis, alternate treatment modalities are being investigated that capitalize on direct manipulations of apoptotic machinery in an effort to reinvigorate apoptotic pathways. Anti-sense oligonucleotides and peptide inhibitors of IAP's have been shown to de-repress apoptotic function (Bilim 2003; Yang 2003A; Yang 2003B; Huang review 2004). Structural analysis of XIAP has also elucidated that the SMAC/DIABLO-binding region can serve as a potential target site for small molecule drug screening used for the treatment of cancers that overexpress IAPs (Liu 2000; Wu 2000; Schimmer, Review 2004B).

Previous studies have demonstrated that various drug resistant prostate cancer cell lines are resistant to TRAIL-induced apoptosis due to overexpression of XIAP. However, surprisingly, treatment with low concentrations of chemotherapeutic drugs sensitized the cells to TRAIL-induced apoptosis through downregulation of XIAP expression (Nimmanapali 2001; Zisman 2001; Eid 2002). The role of XIAP in TRAIL resistance was corroborated by transfection with SMAC/DIABLO, which inhibited XIAP and sensitized the cells to TRAIL (Ng and Bonavida 2002B). Immunotherapy with TRAIL or with agonist monoclonal antibodies to DR4 or DR5 is currently being examined clinically in certain cancers and may be considered for prostate cancer. However, most prostate cancers are resistant to TRAIL-induced apoptosis and require sensitization to reverse the resistance. Evidence suggests that overexpression of XIAP in individual prostate cancers may be a useful marker to immediately identify patients who could benefit from TRAIL or other death ligand-mediated immunotherapy.

The objective of this study is to assess the potential clinical significance of XIAP in human prostate cancer. We utilized immunohistochemistry on ex vivo tissue collected in a tissue microarray platform to examine the association of protein-level expression with clinical progression and prognosis. This large patient cohort enables independent validation of the findings of Krajewska et al. (2003), extending their prognostic findings to include patients who underwent radical prostatectomy as primary therapy, and examining both local as well as advanced cases.

Below paragraph belongs in Discussion:
Here we report that XIAP is elevated in prostate cancer and PIN and is an independent predictor of cancer recurrence, in agreement with previous studies (Ferriera 2001B;

Krajewska 2003). This finding provides further evidence that XIAP expression produces a counterintuitive direct association between expression and favorable clinical outcome implicating an as yet undetermined set of co-regulated mechanisms in this disease model. Nonetheless, the strong associations of XIAP expression to prostate cancer recurrence identifies it as a key molecule for targeted therapeutic investigation.

MATERIALS AND METHODS
Patients
The study cohort consisted of 226 randomly selected hormone naïve patients who underwent radical retropubic prostatectomy between 1984 and 1995. All prostate tumors were staged according to the 1997 American Joint Committee on Cancer TNM staging system (Fleming 1997) and histologically graded using the Gleason scoring system (Gleason 1974). All cases were of the histological type "adenocarcinoma, conventional, not otherwise specified" (Young 2000). Of the 226, 192 were informative for both recurrence outcomes and marker expression data. Table 1 shows the clinicopathologic data for this cohort. The median age at the time of surgery was 65 (range 46 to 76). 112 (58%) patients were low grade (Gleason score 2-6); 80 (42%) were high grade (Gleason score 7-10). 34 patients (18%) had seminal vesicle invasion (pT3b). Concurrent regional lymphadenectomy accompanied 190 (99%) cases, only 11 of which (6%) were positive for metastases. 130 (68%) patients were margin negative, 62 (32%) were margin positive. Regarding capsular invasion, 40 (21%) had no invasion, 113 (59%) had invasion, and 39 (20%) had capsular extension. Approximately half of the tumors, (52%) were confined to the prostate (organ confined here = T2a or T2b with negative lymph nodes, no capsular extension and with negative surgical margins). 38 (20%) patients were considered high risk based on seminal vesicle and/or nodal positivity. The maximum pre-operative serum PSA was known for 172 patients (91%), with a median value of 9.2 ng/ml, (range 0.6-96.5).
A retrospective analysis for outcome assessment was based on detailed anonymized clinicopathologic information linked to the TMA tissue specimens. Recurrence, defined as a postoperative serum PSA of 0.2 ng/ml or greater, was seen in 69 (36%) patients. Total follow-up, defined as the time to recurrence or to last contact in non-recurring patients, had a median of 48.5 months (range 0.1-163). The median overall follow-up, defined as the time from primary surgery to last PSA follow-up, was 78.5 months (range 0.1-182).

Prostate Tissue Microarray (TMA) Construction
Formalin-fixed, paraffin-embedded archival tumor specimens were obtained from the UCLA Department of Pathology under IRB approval. Case material was reviewed for tissue array construction by a study pathologist (DS). At least 3 core tissue biopsies (each 0.6 mm in diameter) were taken from morphologically representative regions of each prostate tumor and precisely arrayed as previously described (Kononen 1998). Tumor samples were accompanied by matching benign (morphologically normal or hypertrophic) and in situ neoplastic lesions (PIN), when available. Case material was arrayed into 3 TMA blocks. For staining, sections (5 um) were transferred to glass slides using an adhesive slide system (PSA-CS 4×, Instrumedics Inc., Hackensack, New Jersey) to support cohesion of the array elements.

Immunohistochemistry

Immunohistochemical staining was performed using an affinity-purified polyclonal rabbit anti-human/mouse XIAP antibody (R&D Systems, Inc. Minneapolis, MN, USA; catalog number AF822; Immunogen: aa 244 - 263 of human XIAP). A standard 2-step indirect avidin-biotin complex (ABC) method was used (Vector Laboratories, Burlingame, CA). Following deparaffinization in xylenes, the array sections were rehydrated in graded alcohols and endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed for 25 minutes in a 95° C solution of 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval, and then blocked with 5% normal goat serum for 30 minutes. The primary antibody was then applied at a1 ug/ml final concentration and incubated at 4°C overnight. After washing, biotinylated goat anti-rabbit IgG was applied for 30 minutes at room temperature. The ABC complex was applied for 25 minutes followed by the chromogen diaminobenzidine (DAB) for 3 minutes. PBS (10 mM, pH 7.4) was used for all wash steps and dilutions. Incubations were performed in a humidity chamber. The sections were counterstained with hematoxylin, followed by dehydration and mounting. PC-3 cells were used as a positive staining control for XIAP. As a negative assay control, pooled non-immune rabbit IgG was applied at the same concentration as the anti-XIAP antibody. Preparation and staining of PC3 cells requires description, and any western blot?

Scoring of Immunohistochemistry

Semi-quantitative assessment of antibody on the TMAs was performed by a study pathologist (HY) blinded to the clinopathological variables. The target tissue for scoring was the glandular prostatic epithelium, scoring of benign tissues did not include basal cells. Tissue spot histology and grading was confirmed on the counterstained study slides. XIAP cytoplasmic expression was scored using two measures, intensity on a 0-3 scale (0=negative, 1=weakly positive, 2=moderately positive, 3=strongly positive), and percentage of positively stained target cells (range 0-100% positive) staining at each intensity. To better represent overall protein levels, we combined the frequency and intensity measures into an integrated intensity measure using the following formula: ((% staining at intensity 3*3)+(% staining at intensity 2*2)+(% staining at intensity 1*1))/100. To represent expression within cases, the mean pooled integrated intensity of the invasive tumor spots was used.

Statistical Analysis

The Kruskal-Wallis and Mann Whitney *U* tests were used to determine the significance of XIAP protein expression differences between categorical clinicopathologic prognostic variables. Associations of XIAP expression with continuous covariates were tested with the Spearman correlation. We used the Pearson chi^2 test to examine the association of dichotomized XIAP expression groups versus categorical variables. Recurrence was defined as a rising total PSA >0.2 ng/ml status post prostatectomy, and time to recurrence was calculated from the date of the primary surgery. Patients without recurrence at last follow-up were censored. Kaplan-Meier plots were used to visualize recurrence-free time distributions and the log rank test was used to test for differences between them. To assess which covariates associate with recurrence-free time, we fit both univariate and

multivariate Cox Proportional Hazards regression models. The proportional hazards assumption was verified using Schoenfeld residuals (Harrell 2001). All p values were 2-sided and p <0.05 was considered significant. All statistical analyses were performed using R statistical software (http://www.r-project.org/) and StatView version 5 (SAS Institute Inc., Cary, N.C., USA).

RESULTS

XIAP protein expression in PC3 prostate cancer cell line and human tissues
XIAP expression by IHC in an androgen-independent human prostate cancer cell line, PC3, showed uniform cytoplasmic staining (*but also see nuclear in image-address*) (Figure 1). These findings also established a positive control for subsequent immunohistochemical analyses in whole prostate tissues and tissue microarrays. Replacing primary anti-XIAP antibody with non-immune pooled rabbit IgG at an equivalent concentration showed an absence of staining.

Staining of XIAP was observed in the normal and malignant glandular epithelium, basal cells, and occasionally in stromal fibromuscular cells. As in PC3, in human prostate tissues XIAP is most typically expressed diffusely in the cytoplasm, but occasionally discrete supranuclear staining in coarse clusters or a combination of diffuse cytoplasmic and coarse supranuclear staining is appreciated (Figure 2). Basal cells in normal glands are frequently quite strongly stained, more than the overlying glandular cells. Our scoring of benign epithelium was limited to these glandular cells. Examples of the XIAP staining intensity spectrum are shown in Figure 3.

We next examined the XIAP protein expression distribution stratified by histological category (Figure 4). 223 cases provided epithelium-informative microarray spots. XIAP is elevated in prostate cancer vs. matching benign tissues, and the increase can be seen starting in PIN, BPH is the lowest expresser. The intensity of XIAP protein expression in cells staining by immunohistochemistry as seen in 1,107 informative tissue microarray spots containing benign prostatic hyperplasia (BPH; n=122), morphologically normal prostate (NL; n=252), prostatic intraepithelial neoplasia (PIN; n=48) and invasive prostate cancer (Cancer; n=685) are shown as mean bar graphs (Figure 4A.) and boxplots (Figure 4B.). The mean XIAP expression was significantly higher in cancer (intensity = 1.32) compared to PIN (intensity = 1.08; p=0.019), normal (intensity = 0.78; p<0.0001), and BPH (intensity = 0.57; p<0.0001). XIAP expression in PIN was significantly higher than normal (p=0.010) and BPH (p<0.0001), and expression in normal epithelium was significantly higher than that seen in BPH (p=0.0006). 69% of BPH stained negatively to weakly (intensity <1.0), 53% of normal, 37% of PIN and only 26% of prostate carcinomas.

The intensity of XIAP protein expression in 685 prostate cancer tissue microarray spots stained by immunohistochemistry is shown as mean bar graphs (Figure 5A.) and boxplots (Figure 5B.). The mean XIAP expression intensity for grades 1-2 (n=116), 3 (n=387), 4 (n=149) and 5 (n=33) were, 1.27, 1.31, 1.37 and 1.22, respectively. There are no significant differences in XIAP expression across the spectrum of Gleason grades. The Mann-Whitney U test was used for two-group comparisons resulting in non-significant p-

values for: grades 1-2 vs. 3 (p=0.30), 4 (p=0.10) and 5 (p=0.40); grade 3 vs. 4 (p=0.10) and 5 (p=0.19) and grade 4 vs. 5 (p=0.40). The Spearman correlation coefficient and its corresponding p-value were used to determine the correlation between XIAP protein expression and Gleason grade as a continuous variable (σ = 0.03, p = 0.37).

XIAP Expression and Cancer Recurrence
We next examined the potential association XIAP protein expression with tumor recurrence following radical prostatectomy. Recurrence data was available for 192 XIAP-informative cases. These cases were represented by 609 primary prostate cancer spots, therefore an average of 3.2 spots per patient. Case-level expression was derived by pooling the mean integrated intensities of the spots. The resultant XIAP expression was examined as both a continuous and dichotomized variable. Supervised survival tree analysis applied to recurrence-free time demonstrates an optimal intensity cut-off of 1.8. XIAP expression intensities of >1.8 and ≤1.8 were considered "High" and "Low" XIAP, respectively.

Mean XIAP expression was directly associated with established prognostic factors in Table 1. As a continuous variable XIAP expression was significantly increased in cases with capsular invasion (mean XIAP = 1.38) verses either cases with no invasion (mean XIAP = 1.10) or capsular extension (mean XIAP = 1.16; Kruskall-Wallis P = 0.016). However, when capsular involvement is examined as a continuous variable no significance is seen (Spearman correlation P = 0.45). There was no significant association between the dichotomized XIAP expression groups and capsular involvement, and there was no significant association of either measure with Gleason score, SV invasion (stage T3b), lymph node status, surgical margins, capsular involvement, overall organ confinement, high-risk groups and preoperative PSA. The dichotomized XIAP group was associated with recurrence status by logistic regression (P = 0.0010; 11.78; 95% confidence interval 2.73-50.88). Despite having a longer overall PSA follow-up, 94% of patients with high XIAP expression were recurrence-free, versus 58% of patients with low XIAP.

The results of Cox proportional hazards analyses performed for established prognostic factors for time to PSA recurrence are shown in Table 2. For all patients as a group, a Gleason score ≥7 (p <0.0001), SV invasion (stage T3b) (p <0.0001), increasing capsular involvement (p = 0.0038) and a higher preoperative PSA (p = 0.015), were all associated with an increased risk of tumor recurrence, whereas a higher level of XIAP expression predicted a reduced risk of tumor recurrence both as a continuous (P = 0.033) and a dichotomized (p = 0.0010) variable in univariate analysis. The dichotomized XIAP remains highly significant in multivariate analysis in this category (P = 0.0025), as well as after substratifying by Gleason score (P = 0.010 for high grade cases). In low grade patients the findings were also striking because no patients (0%) with high XIAP (n=23) recurred, while 26% with low XIAP (n=89) did recur. We were unable to generate survival statistics due to this intriguing lack of event failures in patients with high XIAP expression.

Also of particular note is the strength of XIAP predictive power as a dichotomized variable, as defined by the hazard ratio, which was higher in all cases than the hazard ratios from the conventional prognosticators. In fact, in high grade tumors only XIAP and seminal vesicle invasion remained significant, and in low grade tumors only high XIAP defined a 100% recurrence free subpopulation (in comparison to other low-risk situations, e.g. lack of SV invasion and no capsular involvement defined 81% and 93% recurrence-free subpopulations, respectively. A preoperative PSA within the lowest 10% [0.6-10.2 ng/ml] yielded an 86% recurrence-free subpopulation).

Figure 6A. shows a Kaplan-Meier estimate of cancer recurrence-free time for all 192 outcome-informative patients (Log Rank p <0.0001) stratified by XIAP expression. The median recurrence-free time was 75 months for cases with low XIAP, compared to >152 months for cases with high XIAP. Moreover, only 6% of the high XIAP cases had a tumor recurrence (94% were censored), compared to 42% of the low XIAP cases (58% were censored). When the study is further substratified by either Gleason score, (Figure 6B.), or organ confinement, (Figure 6C.), XIAP remains a powerful predictor. High XIAP portends an optimally good outcome whatever the grade or organ confinement status. Patients with high grade or non-organ confined tumors with high XIAP do better as a group than any patients whose tumors express low XIAP, even those of low grade or that are organ confined. As mentioned above these high XIAP substrata generate subgroups without recurrence, and therefore no Cox or Kaplan-Meier P-values can be calculated from them in these statistical models. However, Table 3 shows how effectively XIAP stratification can isolate low recurrence groups in all patient substrata examined. For example, in patients whose tumors were not organ confined (n=92), 50% experienced disease recurrence. However, within this group, none of the 12 patients with high XIAP expression tumors experienced recurrence, while 58% of the 80 patients with low XIAP expression tumors did recur.

The robustness of the XIAP expression cutoff point was examined by testing cut-off values across the full spectrum of potential XIAP intensities using 0.1 intervals from 0.0-3.0 (Figure 7). As previously noted, the maximum hazard ratio occurs where XIAP is dichotomized at an intensity of 1.8. However, statistical significance is seen across a broad range of XIAP intensity cut-offs (1.3 to 1.9). This range encompasses 73 patients (38% of total patients) and therefore is notable not only for the implied strength of XIAP prediction in cases outside of the gray zone, but also for the potential for misclassification of patients while choosing gray zone cut-offs. Of interest, 1.3 is the *median* expression value for XIAP, and therefore this commonly utilized empirical cut-off point also yields a significant result.

DISCUSSION
I have abbreviated and also moved around some paragraphs for the flow.

Intact apoptotic machinery is critical to cellular homeostasis. Overexpression of anti-apoptotic regulatory proteins may facilitate tumorigenesis (Bilim 2003; Krajewska 2003)

and subsequent cancer cell survival supporting tumor growth (Denmeade 1996), viability during metastatic transit (Berezovskaya, 2005) and resistance to hormonal (XXXX), chemo- (Tamm 2000; Asselin 2001; Imoto 2001), radiation (Holcik 2000) and immunotherapies (Ng and Bonavida, 2002;).

We find that the level of XIAP expression is higher overall in prostate cancer as compared to matched benign tissues, with an intermediate expression elevation seen in PIN, suggesting a link between expression and tumor progression. The potential association of XIAP protein expression with clinicopathological parameters across our prostate cancer cohort was examined. When dichotomized optimally, we found that XIAP expression was a notably strong recurrence risk predictor, high expression associating with a substantially reduced risk of recurrence. In fact, XIAP generated a larger hazard ratio than those seen from conventional prognosticators, including Gleason score, tumor stage capsular invasion and preoperative PSA, and remained significant in multivariate analyses, even as some of the traditional predictors fell out of significance. As demonstration of its predictive power, patients with high grade or non-organ-confined tumors whose tumors expressed high levels of XIAP had a lower risk of recurrence than patients with low grade or organ-confined tumors that express low XIAP levels. Also striking, no patients with low grade tumors expressing high XIAP levels recurred, while over a quarter of those with low XIAP expression experienced recurrences. Despite having a longer overall PSA follow-up, 94% of all patients with high XIAP expression were recurrence-free at the end of follow-up, versus 58% of patients with low XIAP tumors. These findings, coupled to the lack of direct association of dichotomized XIAP expression with any of the clinicopathologic variables tested, and its consistent ability to maintain predictive power in various patient substrata, demonstrates its independence and widespread applicability as a prognosticator.

High levels of XIAP were also found to associate with a reduced risk of recurrence in prostate cancer patients (Krajewska 2003). These findings are entirely consistent with our present study. In addition, in this previous study as well as our current one, no correlation of XIAP with tumor grade or stage is seen, eliminating the potential influence of XIAP associating with these strong prognosticators, as is seen with Ferreira et al. (2001B) in regards to tumor stage. However, Krajewska et al. (2003) did find a significant inverse correlation with pre-operative PSA level and offered that as a potential link to the positive outcome seen in high XIAP-expressing patients. However, in the current study we do not see such a correlation, providing weight to the independence of XIAP's predictive power.

These findings are in agreement with other studies suggesting XIAP's role in promoting tumor cell survival. Pathologically elevated XIAP levels have been found in a number of hematologic (Tamm 2000; Byrd 2002; Kashkar 2003; Tamm 2004A; Tamm 2004B; Yamamoto 2004; Nakagawa 2005), vascular (Levkau 2001) and epithelial (Ferreira 2001A; Hoffman 2002; Krajewska 2003; Shiraki 2003; Liu 2004; Krajewska, 2005) malignancies and also in most cell lines of the NCI-60 tumor screening panel (Fong 2000, Tamm 2000). Only rare exceptions to this pattern are noted, for example high

XIAP protein levels were seen in both normal cervical tissues from non-neoplastic hysterectomies, as well as from cervical cancer cases (Liu 2001).

The association of high XIAP expression with an optimal clinical outcome is counterintuitive to expectations that IAP's promote tumor cell survival and these findings are in contrast to several studies showing a negative prognostic association of XIAP to cancer recurrence/remission and/or death. For example, the relevance of XIAP in progression and prognosis in renal cell carcinoma of the clear cell type was examined by IHC on formalin-fixed tissues from 145 RCC's. XIAP expression was found in 95% of clear cell RCC's (Ramp 2004). A significant increase was observed from well (G1) to poorly (G3) differentiated clear cell RCC's and from low (pT1) to advanced (pT3) tumor stages. Log rank tests showed the significant inverse correlation between XIAP expression and tumor aggressiveness as indicated by patient survival. Despite the association with grade and stage, XIAP expression was nonetheless still found to be an independent negative prognostic survival marker in clear cell RCC when these prognosticators were included.

Tamm et al. (2000) examined 76 patients with AML and found that higher levels of XIAP protein correlated with shorter remission durations after chemotherapy (though not reaching significance) and shorter survival ($p=0.5$) in patients with AML. However, in follow-up studies adding 172 additional AML samples the previously seen clinical correlation from Tamm et al. (2000) did not hold (Carter 2003). The authors noted a much higher relapse rate in the earlier patient cohort (70%) versus the newer group (52%), which was felt to be reflective of therapy differences at different time periods, but no other differences in the patient cohorts were found and the authors were not able to provided an explanation for the loss of correlation. Later studies by the same group found that high XIAP levels associated with poor overall survival in both de novo adult and childhood AML in separate studies (Tamm 2004A; Tamm 2004B). In agreement, XIAP positivity, combined with a high caspase 3 activation, proved to be a significant negative survival predictor in B-cell Lymphoma (Muris 2005).

Several other studies failed to find associations between XIAP levels and survival, including those focusing on colon (Krajewska 2005), cervical (Liu 2001) and bladder (Bilim 2003) cancers, the later two studies also noted a lack of association of XIAP with tumor grade and stage.

Conversely, recent studies have demonstrated that increased levels of XIAP are paradoxically associated with an *improved* prognosis (Krajewska 2003; Ferreira et al., 2001B), in agreement with our current study. Ferriera et al. (2001B) examined XIAP expression in 144 radically resected patients with early stage non-small cell lung cancer (NSCLC). They examined XIAP by IHC and found that high XIAP expression associated with a longer overall survival than lower expression ($p=0.01$). The authors found that XIAP expression was associated inversely with cellular proliferation, (measured by the

Ki67 and mitotic indexes) and with tumor stage, providing some explanation for the unexpected positive outcome. But, despite these correlations, XIAP was still found to be an independent predictor in multivariate analysis and therefore its positive impact could not be ascribed solely to associations with known prognosticators.

Interestingly, the same group found that XIAP was not associated with survival in advanced stage, non-resectable NSCLC, despite also being elevated in these tumors (Ferreira 2001A). In addition, no association with tumor stage or grade was seen, suggesting that the influence of XIAP alone may be insufficienet to provide predictive power in certain patient subsets.

There are a number of possible explanations for our findings. XIAP has been reported to mediate cell cycle arrest via downregulation of cyclins A and D1, (possibly through selective ubiquitination by XIAP), and via induction of cyclin-dependent kinase inhibitors p21Cip1/Waf1 and p27Kip1 (Levkau 2001). Thus, while XIAP may provide a selective anti-apoptotic survival advantage it may simultaneously impair proliferation of cancer cells, and it is possible that these two properties function with some independence. For example, Ferreira et al. (2001B) documented decreased proliferation with XIAP expression, but they saw no difference in the apoptotic index between the high and low XIAP groups and therefore apoptotic rate did not appear to be a contributor to tumor cell survival. However, to the contrary, high XIAP in ovarian cancer cell lines occurred in proliferating cells with low apoptosis (Sasaki 2000). Bilim et al. (2003) found no cell cycle promotion in TCC cell lines concommitant with downregulation of XIAP, though apoptosis increased, and Liu et al. (2001) found no association of either apoptosis nor proliferation with higher XIAP in cervical cancers suggesting that the overall effects are complex and may be cell-type, or tumor-specific. The cellular proliferation rate was not examined in the current prostate cancer study nor in the study by Krajewska et al (2003).

However, Krajewska et al. (2003) did examine the concomitant expression of other IAP's. In contrast to XIAP, high expression of cIAP-1 and cIAP-2 correlated with poor recurrence-free survival in these same patients, while results with survivin were inconclusive, suggesting that various IAP's may predominate in certain situations, perhaps providing redundant apoptotic regulation. Several members of the IAP protein family are direct inhibitors of caspases, and their importance as prognostic markers in malignancy has been well documented. For example, the negative prognostic significance of survivin overexpression has been demonstrated in studies examining leukemic (Nakagawa 2005; Tamm 2004A; Tamm 2004B), brain (Kajiwara 2003), colon (Sarela 2000), gastric (Miyachi 2003), urothelial (Schultz 2003) and hepatocellular (Ikeguchi 2002) cancers. However, not all studies examining survivin are in agreement (Carter 2003; Reed and Bischoff Review 2000).

XIAP expression is regulated by a number of cell survival pathways. Studies have found that Nuclear Factor kappa-B (NFKB), which may be induced by a number of cellular stresses and is associated with tumorigenesis, induces expression of cIAP-1, cIAP-2 and XIAP in some cells (Stehlik 1998; Sonoda 2000). Interestingly, XIAP can help induce transcriptional activation of NFKB through phosphorylation (via activation of the IkB

kinase complex, IKK) and subsequent proteasomal degradation of IkB inhibitory proteins, ultimately activating NFkB target genes, including XIAP. Therefore there is a positive feedback loop thereby facilitating NFKB-mediated cell survival (Hofer-Warbinek 2000; Levkau 2001). Carter et al. (2003B) have found that XIAP is regulated by cytokines through PI3K, and to a lesser degree through MAPK pathways. The induction of XIAP expression by cytokines through PI3K/MAPK pathways is consistent with its role in cell survival.

Translation of XIAP is controlled by a rare 162-nucleotide IRES element located in the 5' unstranslated region of XIAP mRNA, a sequence critical for cap-independent translation that facilitates its antiapoptotic function during any kind of induced-cellular stress such as radiation and chemotherapy (Holcik 1999; Holcik 2000; Holcik Review 2003; Holcik 2005; Lewis 2005). This mechanism provides resistance to translation repression normally accompanying cellular stress, providing cells an increased chance at survival until stresses abate, but also resulting in treatment resistence. Since post-transcriptional regulation of XIAP is controlled by an IRES site, differences have been seen between mRNA and protein levels in some cell lines (Tamm 2000). However, good correlations between the two have been seen in RCC and NSCLC (Yan 2004 and Hofmann 2002, respectively) examined by RT-PCR and Western blot.

XIAP, like some other IAP's, also contains a zinc-binding (finger) motif, the RING domain, that allows the protein to act as an E3 ubiquitin ligase that binds ubiquitin-conjugating enzymes promoting degradation of IAP-caspase complexes. Therefore, as well as regulating caspases, XIAP is itself regulated by ubiquitin-mediated degradation. (LaCasse 1998; Yang 2000; Salvesen REVIEW 2002; Vaux 2005).

Moreover, XIAP is itself regulated by the co-expression in the tumor cells of endogenous antagonist proteins that can negate apoptosis suppression by XIAP. For example, apoptosis is promoted by the release of Smac/DIABLO from the mitochondria into the cytosol where it directly binds to the BIR domains of IAP's blocking caspase inhibition (Du 2000; Verhagen 2000; van Gurp 2003). Overexpression of Smac/DIABLO can sensitize cancer cells to TRAIL-induced apoptosis (Ng 2002B; Guo 2002; Mizutani 2005) and therefore it is a major agonist of XIAP. Abnormally high levels of IAPs commonly found in cancer cells can prevent Smac/DIABLO from carrying out its function (LaCasse 1998). A recent study of renal cell carcinomas by Yan et al. (2004) found a delicate balance between XIAP and Smac/DIABLO, suggesting that it is the *relative* increase of XIAP over Smac/DIABLO that promotes apoptosic inhibition.

Like Smac/DIABLO, HtrA2/Omi is released from the mitochondria into the cytosol as a result of pro-apoptotic stimuli, where it binds to XIAP, displacing caspases as with other anti-IAP's (Hedge 2002; Verhagen 2002). XIAP associated factor-1 (XAF1) is another cytosol protein that effectively blocks XIAP's anti-apoptotic activity (Fong 2000; Liston 2001) and its downregulation has been associated with malignancy (Fong 2000; Byun 2003; Ng 2004). The mechanism of action may be in the redistribution of XIAP from the cytoplasm to the nucleus (Liston 2001). In our study we saw rare nuclear staining, as did

Liu et al. (2001), though in most published studies nuclear localization of XIAP is not noted (Ferreira 2001A; Ferreira 2001B; Bilim 2003; Shiraki 2003; Ramp 2004).

The present study has weaknesses of note. As with all IHC-based studies, we do not know the activity and overall structural integrity of the detected XIAP protein, which may be influenced by posttranslational modification. We also do not know if its association with other proteins at the time of tissue fixation might sterically hinder antibody detection of target epitopes. In addition, all of the histomorphologically benign tissues in the study derive from cancer patients. Findings of genetic, epigenetic and induced aberrations in expression in normal-appearing tissues located adjacent to tumors has been reported (Chandran 2005). In an effort to ameliorate this potential, we extracted benign tissue cores from non-tumor containing donor blocks, or at maximal distances from tumors in the same block, wherever available.

To our knowledge, this study includes the largest single cancer patient cohort to date examining the XIAP protein by in situ IHC for association to clinical outcomes. Our findings are in strong agreement with those seen by Krajewska et al. (2003) and provide independent validation for the association of high XIAP protein expression and lower recurrence risk in prostate cancer. The patient cohort for clinical outcomes in the aforementioned study was limited to needle core biopsies from 64 T2N0M0 radiation-treated patients. Here we provide an expanded and unrelated patient population on tissue microarrays to include 192 informative patients with a spectrum of disease stages and report for the first time p-values and hazard ratios across all possible cut-points in XIAP expression (figure 7).

There are currently no biomarkers that can consistently predict response to conventional therapies in prostate tumors. Since caspase 3 and 9 have been shown to be involved in chemotherapy-induced apoptosis, and caspase 3 and 8 are involved in receptor-induced apoptosis (Sun 1999), it is expected that XIAP may be used to predict therapeutic response. However, it is clear that XIAP works in delicate balance with other apoptosis pathway proteins and therefore a more complete understanding of its influence requires future multimarker studies including other IAP's and anti-IAP's as well as studies of the integrity of caspase 8 versus caspase 9 pathways in each patient, including FLIP, p53 and Bcl2, in order to clarify predictions.

Since IAPs are preferentially expressed in malignant cells and may be prognostically important, they are also attractive therapeutic targets, and efforts are being made to develop promising IAP inhibitors for clinical use which also minimize or prevent harm to normal tissues. However, the benefit of specifically targeting XIAP for such sensitization has been questioned because of evidence that its function is redundant. For example, XIAP knockout mice were viable, underwent normal tissue development and had retained apoptosis (Harlin 2001). In the absence of XIAP, levels of some other IAP's were increased, suggesting compensatory regulatory mechanisms. In addition, Ferreira et al. (2001A) found no association between XIAP expression and response to chemotherapy in advanced NSCLC's, and despite being a negative clinical prognosticator in AML, Tamm et al. (2000) found that higher XIAP unexpectedly correlated with

chemosensitivity in NCI-60 tumor cell lines, while cIAP-1 correlated, as expected, with chemoresistance in the same model.

With empasis on XIAP's central role, antisense oligonucleotide technology targeted at XIAP transcripts has been shown to both reduce XIAP protein expression and concomitantly increase human cancer cell response to chemo- and immunotherapies and radiation as evidenced by increased apoptosis in cell lines and xenografts. Selective downregulation of XIAP expression in this manner has resulted in resensitization of a variety of tumor cell types, including leukemia (Carter 2003B), lung (Holcik 2000; Hu 2003), ovarian (Sasaki 2000); bladder (Bilim 2003), gastric (Tong 2005), breast (McManus 2004) and prostate cancers (Berezovskaya 2005). Because of its potential clinical utility, recently a second-generation antisense oligonucleotide has entered Phase I clinical trials in the United Kingdom (Cummings 2005).

Furthermore, peptide and non-peptide small molecule inhibitors of XIAP function have been shown to induce apoptosis in target cells as well (Tamm 2003). Smac/DIABLO agonists with functional XIAP-binding short peptide have been shown to increase TRAIL-mediated (Fulda 2002; Guo 2002) and chemotherapeutic (Arnt 2002; Yang 2003B) cell killing. In addition, small-molecule derivatives of polyphenylurea, screened for efficacy in sterically hindering caspase-binding to XIAP and therefore functioning as Smac/DIABLO mimetics, have demonstrated in vivo antitumour activity against human prostate and colon cancer xenografts in the absence of significant toxicity to normal tissues (Kipp 2002; Schimmer 2004A; Schimmer, Review 2004B; Wang 2004). As expected, small molecule Smac/DIABLO mimetics have also been found to potentiate TRAIL-mediated cell death (Li 2004).

Interestingly, while therapeutic doses of chemotherapy and radiation cause upregulation of XIAP (Holcik 2000; Ng 2002) we have previously shown that low doses of chemotherapy actually sensitize cells to TRAIL-mediated killing without the use of the antiapoptotic modalities mentioned above (Ng 2002A).

CONCLUSIONS

Malignant prostate cancer remains a disease with few useful outcome measures and no current consistently effective therapies. Therefore, informative biomarkers are urgently needed to guide patient surveillance and clinical intervention. This study reports the overexpression of XIAP in primary human prostate cancers and provides strong evidence for its couterintuitive beneficial prognostic association, in agreement with previous reports (Ferreira 2001B; Krajewska 2003). Our results support the general concensus that XIAP is a reasonable target for intervention to reverse drug and immune resistance. However, given the improved prognosis in high expressors of XIAP there is an unresolved question of the ultimate benefit of anti-XIAP therapeutics in these tumor types and further investigation with this focus is suggested in these, as well as other tumor types. (???)

XIAP is a promising diagnostic, prognostic and therapeutic factor in prostate cancer. While this study was based on examining the expression of XIAP by immunohistochemistry, other methods could also be applied, such as RT-PCR, Western, ELISA, and many other methods available for detection.

Therefore, even though XIAP is the strongest caspase inhibitor, a more complete understanding of IAP contribution to patient outcome may only follow from knowledge of global IAP levels.

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TABLES

Table 1. Relationship of XIAP protein expression with clinicopathologic parameters in prostate adenocarcinomas.

	All Patients	Mean XIAP Expression (Standard Error)	P-value[a] (XIAP as a Continuous Variable)	"Low" XIAP Intensity ≤ 1.8 (% of Total)	"High" XIAP Intensity > 1.8 (% of Total)	P-value[b] (XIAP as a Dichotomized Variable)
Total Cases (n=192)		1.28 (0.041)		**158** (82)	**34** (18)	
Age At Surgery						0.26 (NS)[a]
Median (Range)	65 (46-76)			65 (46-76)	63.0 (50-75)	
Mean	63.8			64.0	63.0	
Gleason Score			0.99 (NS)			0.31 (NS)
2-6	112 (58)	1.28 (0.055)		89 (56)	23 (68)	
7-10	80 (42)	1.27 (0.063)		69 (44)	11 (32)	
Pathology pT Stage[c]			0.63 (NS)			0.21 (NS)
PT2-pT3a	158 (82)	1.28 (0.046)		127 (80)	31 (91)	
PT3b	34 (18)	1.24 (0.092)		31 (20)	3 (9)	
Lymph Node Status (n=190)			0.47 (NS)			>0.99 (NS)
Positive	11 (6)	1.12 (0.202)		9 (6)	2 (6)	
Negative	179 (94)	1.29 (0.042)		147 (94)	32 (94)	
Surgical Margins			0.36 (NS)			0.55 (NS)
Positive	62 (32)	1.22 (0.076)		53 (34)	9 (26)	
Negative	130 (68)	1.30 (0.049)		105 (66)	25 (74)	
Capsular Involvement			**0.016[d]**			0.11 (NS)
No Invasion	40 (21)	1.10 (0.094)		34 (21)	6 (18)	
Invasion	113 (59)	1.38 (0.052)		88 (56)	25 (73)	
Extension	39 (20)	1.16 (0.090)		36 (23)	3 (9)	
Organ Confined[e]			0.15 (NS)			0.15 (NS)
Yes	100 (52)	1.33 (0.058)		78 (49)	22 (65)	
No	92 (48)	1.22 (0.058)		80 (51)	12 (35)	

High Risk[f] (n=190)			0.62 (NS)			0.28 (NS)
Yes	38 (20)	1.24 (0.090)		34 (22)	4 (12)	
No	152 (80)	1.29 (0.046)		122 (78)	30 (88)	
PreOpPSA ng/ml (n=172)						0.80 (NS)[a]
Median (Range)	9.2 (0.6-96.5)			9.8 (0.6-76.0)	8.9 (3.2 –96.5)	
Mean	14.0			14.0	14.0	
<10	87 (51)	1.31 (0.063)	0.74 (NS)			0.48 (NS)
≥10	85 (49)	1.31 (0.061)				
Recurrence[g]			0.082 (NS)[h]			**0.0010[h]**
Yes	69 (36)	1.18 (0.059)		67 (42)	2 (6)	
No	123 (64)	1.33 (0.055)		91 (58)	32 (94)	
Overall Follow-up[i], months						0.085 (NS)[a]
Median (Range)	78.5 (0.1-182.0)			74.0 (0.1-182.0)	88.5 (6.0-152.0)	
Mean	74.5			72.4	84.2	
Total Follow-up[j], months Median (Range)						**<0.0001[a]**
Mean	48.5 (0.1-163.0) 52.3			41.0 (0.1-163.0) 46.1	87.0 (6.0-152.0) 81.3	

[a] P-value was determined by the Mann-Whitney *U* test unless otherwise specified.

[b] P-value was determined by the Pearson chi-squared test with Yates continuity correction unless otherwise specified

[c] pT3b indicates seminal vesicle invasion. There are no pT4 cases.

[d] P-value was determined by the Kruskal-Wallis test. With capsular involvement as a continuous variable, P=0.45 using the Spearman correlation corrected for ties.

[e] Organ Confined = no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative.

[f] High-Risk = seminal vesicle and/or nodal positivity.

[g] Recurrence = PSA elevation raising >0.2 ng/ml status post radical prostatectomy

[h] XIAP mean intensity association with recurrence by logistic regression of continuous data; (P=0.082; 0.63; 95% confidence interval 0.37-1.06), and of dichotomized data (P=0.0010; 11.78; 95% confidence interval 2.73-50.88). XIAP expression was the independent variable.

[i] Overall Follow-up = time from primary surgery to last PSA follow-up.

[j] Total Follow-up = time to recurrence or last follow-up in non-recurrence.

Table 2. Cox Proportional Hazards analysis for time to PSA recurrence.

Variable	Univariate[a] (All Patients[b], n=192)	Multivariate[a] (All Patients[c], n=172)		Univariate[a] (Low Gleason Score[d], n=112)	Multivariate[a] (Low Gleason score[e], n=103)		Univariate[a] (High Gleason Score[f], n=80)	Multivariate[a] (High Gleason score[g], n=69)	
		Continuous	Dichotomized		Continuous	Dichotomized		Continuous	Dichotomized
Gleason Score > 7	**<0.0001** 3.70 (2.23-6.11)	**0.0011** 2.81 (1.51-5.24)	**0.0014** 2.80 (1.49-5.26)	NA	NA	NA	NA	NA	NA
Seminal Vesicle Invasion (Stage = pT3b)	**<0.0001** 4.10 (2.47-6.81)	**0.0035** 2.46 (1.35-4.51)	**0.0032** 2.46 (1.35-4.47)	**0.0065** 5.52 (1.61-18.89)	**0.037** 4.07 (1.09-15.20)	m	**0.0086** 2.21 (1.22-3.98)	**0.012** 2.36 (1.21-4.60)	**0.0089** 2.45 (1.25-4.80)
Capsular Invasion	**0.0038** 1.73 (1.19-2.52)	**0.019** 1.67 (1.09-2.57)	**0.036** 1.55 (1.03-2.35)	**0.014** 2.21 (1.17-4.16)	**0.0049** 3.08 (1.41-6.73)	m	0.42 1.23 (0.75-2.04)	0.52 1.20 (0.69-2.09)	0.69 1.11 (0.66-1.86)
Preoperative PSA	**0.015** 1.02 (1.00-1.03)[h]	0.60 1.00 (0.99-1.02)	0.70 1.00 (0.99-1.02)	**0.024** 1.04 (1.01-1.07)[i]	**0.011** 1.04 (1.01-1.08)	m	0.95 1.00 (0.98-1.02)[j]	0.84 1.00 (0.98-1.02)	0.67 1.00 (0.98-1.02)
XIAP intensity (continuous)[k]	**0.033** 1.54 (1.04-2.29)	0.077 1.49 (0.96-2.33)	NA	**0.028** 2.20 (1.09-4.44)	0.17 1.85 (0.77-4.43)	NA	0.25 1.33 (0.82-2.17)	0.19 1.42 (0.84-2.41)	NA
XIAP intensity ≤ 1.8 (dichotomized)[l]	**0.0010** 10.69 (2.61-43.73)	NA	**0.0025** 8.92 (2.16-38.86)	m	NA	m	**0.011** 6.37 (1.54-26.43)	NA	**0.010** 6.61 (1.57-27.89)

[a] P-value; Hazard Ratio; (95% Confidence Interval) provided

[b] 64% of cases are censored

[c] 67% of cases are censored

[d] Gleason Score 2-6; 79% of cases are censored

[e] Gleason Score 2-6; 83% of cases are censored

[f] Gleason Score 7-9 (no Gleason Score 10 cases are present); 43% of cases are censored

[g] Gleason Score 7-9 (no Gleason Score 10 cases are present); 43% of cases are censored

[h] n=172

[i] n=103

[j] n=69

[k] Pooled mean XIAP intensity. Used formula (3-Continuous XIAP intensity) to reverse hazard ratio to compare directly to other covariates. A high XIAP carries a reduced risk of recurrence.

[l] Pooled mean XIAP intensity dichotomized: ≤1.8 (n=158); > 1.8 (n=34)

[m] High XIAP group has no events (all patients are censored)

Table 3. Prostate cancer recurrence status in patient groups and substratified by XIAP protein expression category

Patient Group	Total Count	Total % Censored[a]	Low XIAP[b] % Censored (Count)	High XIAP[b] % Censored (Count)
All Patients	n=192	64%	58% (n=158)	94% (n=34)
Low Grade[c]	n=112	79%	74% (n=89)	100% (n=23)
High Grade	n=80	43%	36% (n=69)	82% (n=11)
Organ Confined[d]	n=100	77%	73% (n=78)	91% (n=22)
Not Confined	n=92	50%	42% (n=80)	100% (n=12)

[a] Proportion of patients who reach the end of PSA follow-up without evidence of recurrence. Recurrence = PSA elevation raising >0.2 ng/ml status post radical prostatectomy.

[b] Pooled mean XIAP intensity dichotomized: Low ≤1.8; High > 1.8 on a 0-3 scale.

[c] Low Grade = Gleason Score of 2-6; High Grade = Gleason Score of 7-9 (there are no cases of Gleason 10 in this cohort.

[d] Organ Confined = no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative.

FIGURES AND LEGEND

Figure 1. XIAP protein expression in PC3 prostate cancer cell line. Application of anti-XIAP antibody by immunohistochemistry demonstrates cytoplasmic staining of XIAP protein (A) (NOTE: WHY ALSO SEEN IN NUCLEI?). Replacing primary anti-XIAP antibody with non-immune pooled rabbit IgG at an equivalent concentration serves as negative control (B), note the absence of staining. (400 X magnification).
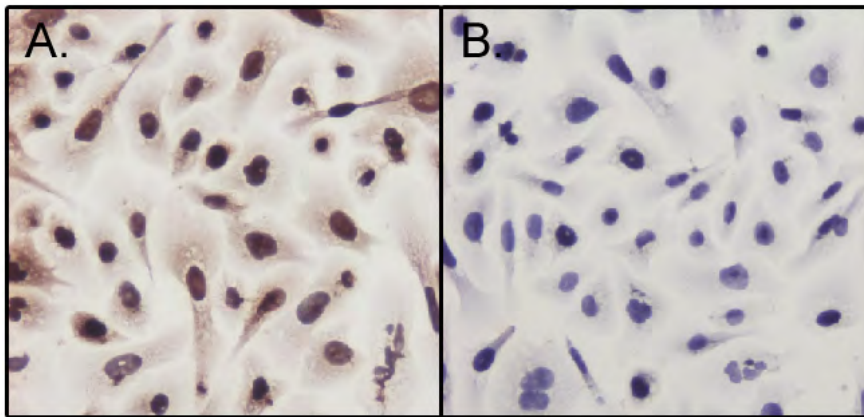
Figure 2. Histocytologic staining patterns of XIAP in prostate epithelium.
Demonstration of the variety of staining pattern of XIAP protein by
immunohistochemistry showing: (A), most typical diffuse cytoplasmic staining; (B),
occasionally appreciated discrete supranuclear staining in coarse clusters and; (C), a
combination of diffuse cytoplasmic and coarse supranuclear staining. (400 X
magnification).
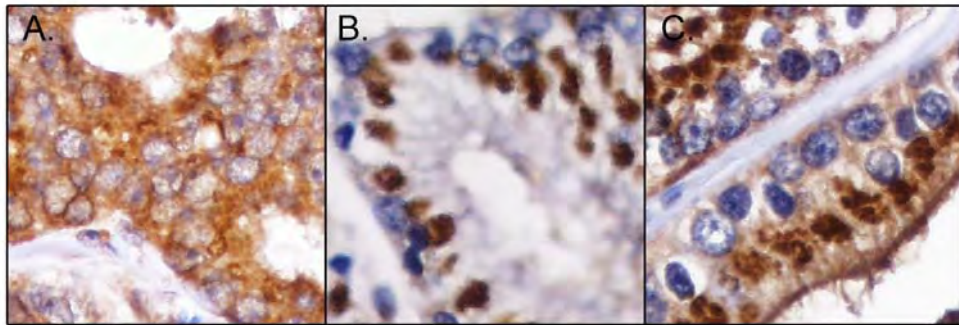
Figure 3. XIAP protein expression in morphologically normal prostate and prostate cancer on tissue microarrays. Immunohistochemical staining for XIAP protein is seen on representative prostate tissue samples. (A) Normal tissue showing weak cytoplasmic epithelial staining of glandular cells. Staining in basal cells is frequently higher than that seen in glandular cells – scoring is from glandular cells. Invasive prostate cancers are shown demonstrating weak (B), moderate (C), and strong (D) cytoplasmic staining. (100X magnification with 400X inserts).
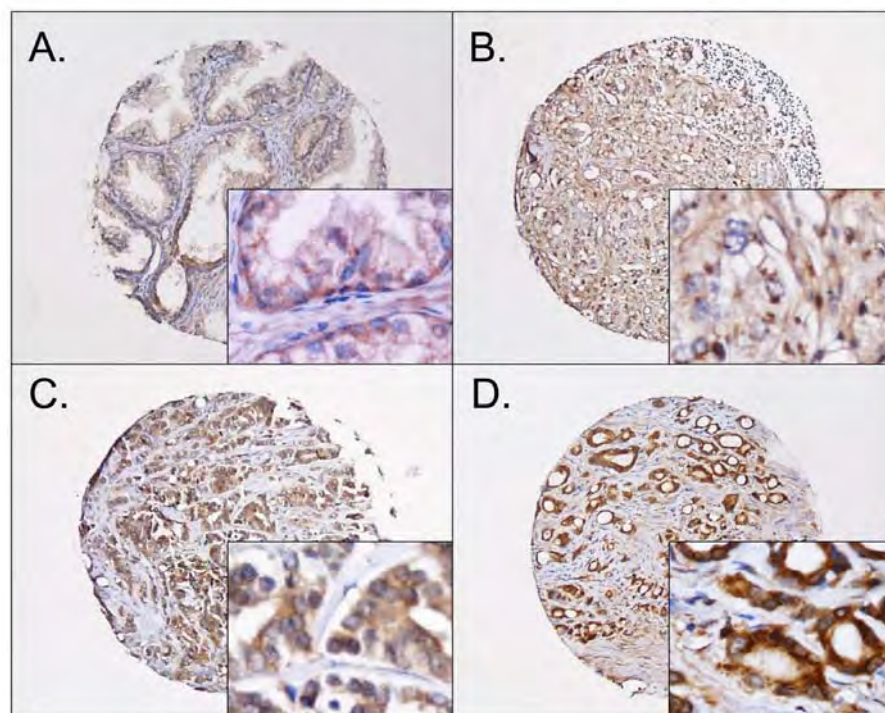
Figure 4. XIAP protein expression distribution on the prostate tissue microarray stratified by histological category. The intensity of XIAP protein expression in cells staining by immunohistochemistry as seen in 1,107 informative tissue microarray spots containing benign prostatic hyperplasia (BPH; n=122), morphologically normal prostate (NL; n=252), prostatic intraepithelial neoplasia (PIN; n=48) and invasive prostate cancer (Cancer; n=685) are shown as mean bar graphs (A.) and boxplots (B.). The mean XIAP expression was significantly higher in cancer (intensity = 1.32) compared to PIN (intensity = 1.08; p=0.019), normal (intensity = 0.78; p<0.0001), and BPH (intensity = 0.57; p<0.0001). XIAP expression in PIN was significantly higher than normal (p=0.010) and BPH (p<0.0001), and expression in normal epithelium was significantly higher than that seen in BPH (p=0.0006). The Mann-Whitney *U* test was used for two-group comparisons. Error bars in A. represent 1 standard error.

A.

B.
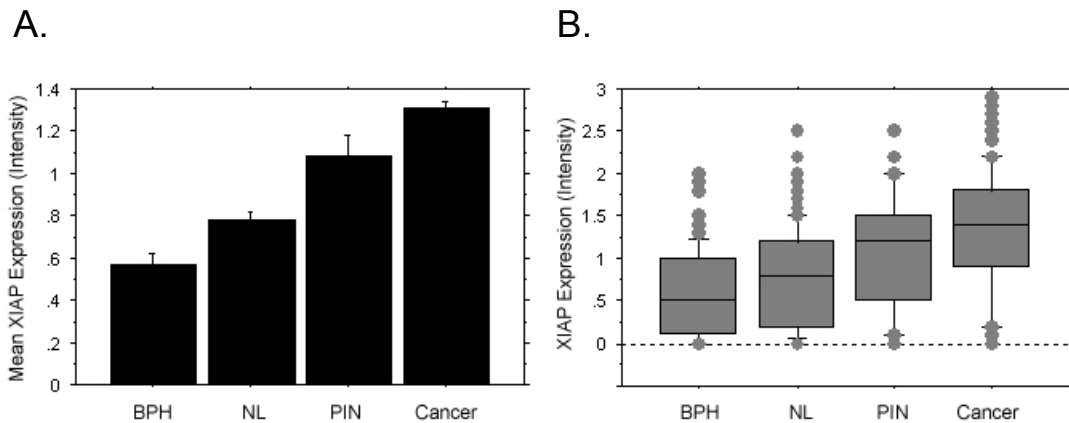
Figure 5. XIAP protein expression distribution of prostate cancers on the tissue microarray stratified by Gleason Grade. The intensity of XIAP protein expression in 685 prostate cancer tissue microarray spots stained by immunohistochemistry is shown as mean bar graphs (A.) and boxplots (B.). The mean XIAP expression intensity for grades 1-2 (n=116), 3 (n=387), 4 (n=149) and 5 (n=33) were, 1.27, 1.31, 1.37 and 1.22, respectively. There are no significant differences in XIAP expression across the spectrum of Gleason grades. The Mann-Whitney *U* test was used for two-group comparisons resulting in non0-significant p-values for: grades 1-2 vs. 3 (p=0.30), 4 (p=0.10) and 5 (p=0.40); grade 3 vs. 4 (p=0.10) and 5 (p=0.19) and grade 4 vs. 5 (p=0.40). The Spearman correlation coefficient and its corresponding p-value were used to determine the correlation between XIAP protein expression and Gleason grade as a continuous variable ($\sigma = 0.03$, $p = 0.37$). Error bars in A. represent 1 standard error.

A.

B.
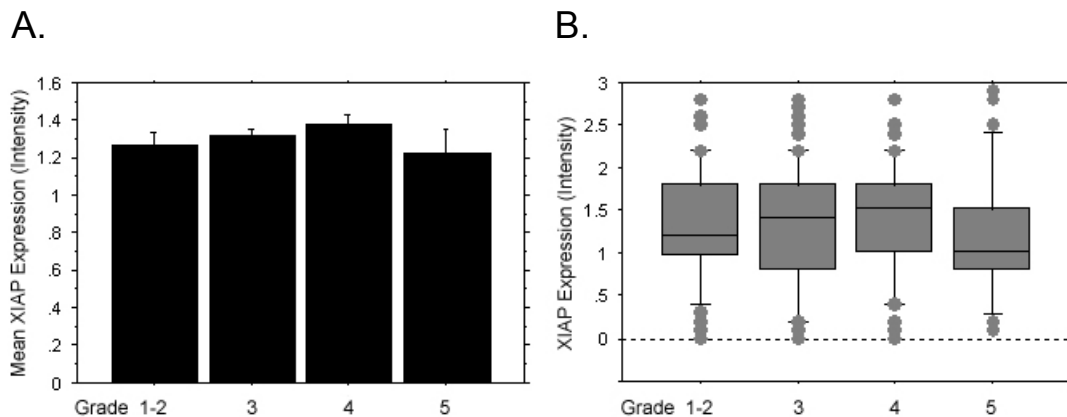
Figure 6. Kaplan-Meier curves for time to prostate cancer recurrence. Kaplan-Meier Curves for time to tumor recurrence stratified by cytoplamsic XIAP protein expression status (n=192 patients) are seen in all patients (A.), and in patients stratified by tumor grade (B.) or organ confinement (C.). XIAP expression intensities of >1.8 and ≤1.8 are considered "High" and "Low" XIAP, respectively. Gleason Scores of 7-10 and 2-6 are considered "High" and "Low" grade, respectively. Cases without capsular extension and/or seminal vesicle and/or lymph node involvement, and with negative surgical margins, are considered organ "Confined", otherwise, "Not Confined". The high cytoplasmic XIAP expression phenotype is consistently associated with a lower risk of developing recurrent prostate cancer. Censored times marked by either circles or triangles.
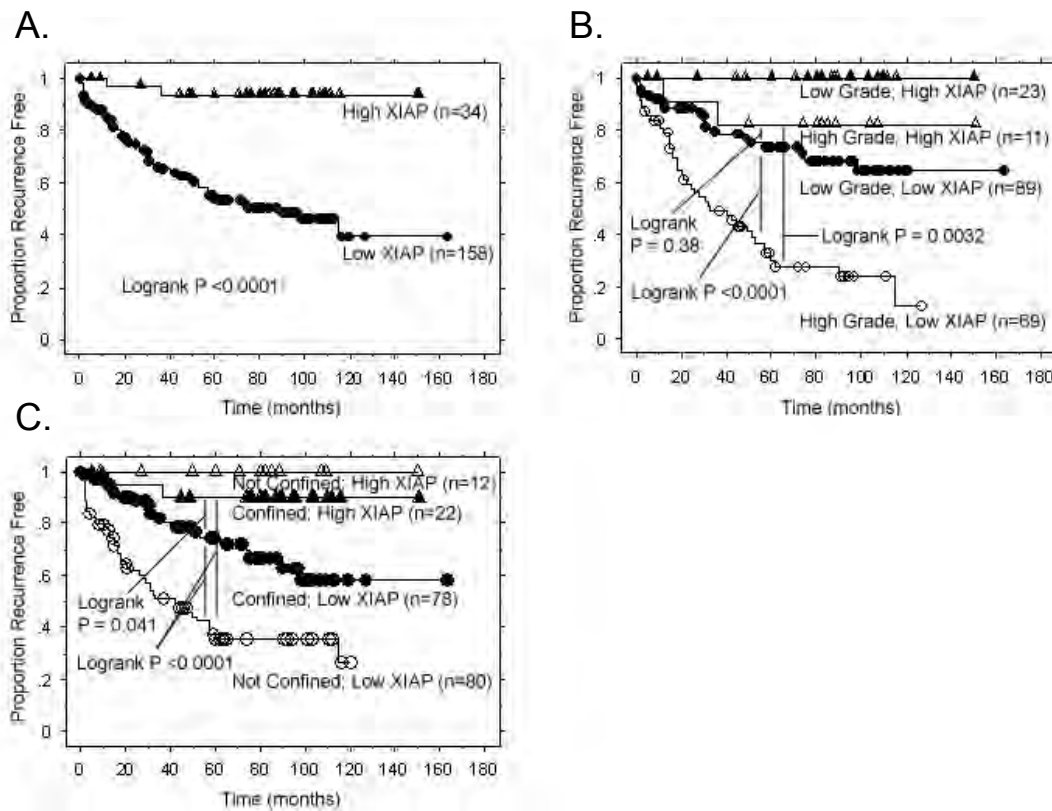
Figure 7. Examination of XIAP intensity cut-off points using Cox proportional hazards model. Cox proportional hazards model for prostate cancer recurrence is depicted here across the full spectrum of potential XIAP intensity cut-off points (using "≤" and 0.1 intervals from 0-3.0). Lines depict resulting P-Values (solid), and hazard ratios (dashed). The gray box encompasses the region where the P-value is significant ($P<0.05$). The maximum hazard ratio occurs where XIAP is dichotomized at an intensity of 1.8, and is significant, (and with a hazard ratio > 1.0), between 1.3 and 1.9. 1.3 is the median expression value for XIAP. At cut-off points above 2.4, the model becomes uninformative due to a lack of recurrences in the high XIAP group and at points above 2.8 because no higher staining is seen.
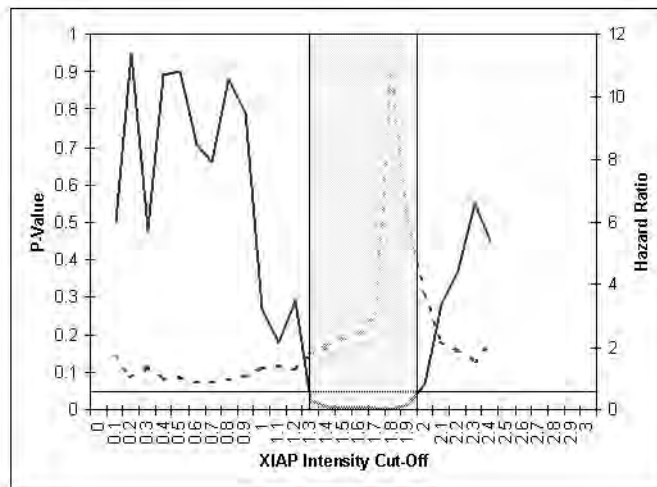
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